Two knockdown models of the autism genes \textit{SYNGAP1} and \textit{SHANK3} in zebrafish produce similar behavioral phenotypes associated with embryonic disruptions of brain morphogenesis.

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ABSTRACT

Despite significant progress in the genetics of autism spectrum disorder (ASD), how genetic mutations translate to the behavioral changes characteristic of ASD remains largely unknown. ASD affects 1-2% of children and adults, and is characterized by deficits in verbal and non-verbal communication, and social interactions, as well as the presence of repetitive behaviors and/or stereotyped interests. ASD is clinically and etiologically heterogeneous, with a strong genetic component. Here, we present functional data from syngap1 and shank3 zebrafish loss-of-function models of ASD. SYNGAP1, a synaptic Ras GTPase activating protein, and SHANK3, a synaptic scaffolding protein, were chosen because of mounting evidence that haploinsufficiency in these genes is highly penetrant for ASD and intellectual disability (ID). Orthologs of both SYNGAP1 and SHANK3 are duplicated in the zebrafish genome and we find that all four transcripts (syngap1a, syngap1b, shank3a and shank3b) are expressed at the earliest stages of nervous system development with pronounced expression in the larval brain. Consistent with early expression of these genes, knockdown of syngap1b or shank3a cause common embryonic phenotypes including delayed mid- and hindbrain development, disruptions in motor behaviors that manifest as unproductive swim attempts, and spontaneous, seizure-like behaviors. Our findings indicate that both syngap1b and shank3a play novel roles in morphogenesis resulting in common brain and behavioral phenotypes.
INTRODUCTION

Autism spectrum disorder (ASDs) is a heritable neurodevelopmental disorder diagnosed in 1-2% of children and adults worldwide (1). ASD core phenotypic features include social deficits and stereotyped behavioral patterns (2). While many contributing genetic loci have been identified, hundreds of other rare genetic disruptions are likely to contribute to ASD, making this disorder genetically heterogeneous (3-11). Moreover many ASD-associated genes have been linked to multiple, often comorbid, neurological disorders including epilepsy and intellectual disability (12-15), indicating that specific combinations of genetic mutations (16, 17), polygenic background (18), genetic-environmental interactions (19-21), or other factors determine how DNA alterations manifest clinically.

To explore how diverse genetic disruptions linked to ASD in humans may converge on common neuropathology, we sought to compare animal models of two genes linked to ASD: SYNGAP1 (*synaptic Ras GTPase activating protein 1*) and SHANK3 (*SH3 and multiple ankyrin repeat domains 3*). In humans, heterozygous mutations in either SYNGAP1 or SHANK3 produce partially overlapping clinical pathologies that include epilepsy, intellectual disability and ASD (4, 22-35). Similarly, both Syngap1 and Shank3 loss-of-function mouse models have overlapping phenotypes that include seizures, increased stereotyped behavior and spatial memory deficits (36-41). Consistent with convergent phenotypes, Syngap1 and Shank3 proteins are both enriched at glutamatergic post-synaptic densities (PSDs) and interact directly in yeast two-hybrid assays (42). Despite these commonalities, Syngap1 and Shank3 loss-of-function mutations cause divergent functional synaptic deficits: decreasing murine Syngap1 causes precocious
maturation of synapses and increased synaptic transmission (37, 43) while decreasing murine Shank3 reduces the density and size of PSDs and decreases synaptic transmission (36, 39, 40, 44). Given similar ASD-related behavioral phenotypes, we wanted to test the hypothesis that Syngap1 and Shank3 have other non-synaptic roles that might provide convergent disease mechanisms.

Zebrafish (Danio rerio) models complement extant mouse models by providing insight into how genes linked to ASD impact early neural circuit development (45, 46). In contrast to mammals, optically transparent zebrafish develop externally and gene knockdown technologies are inexpensive and rapid (47). Previous zebrafish models of ASD genes, such as zdisc1, met and chd8 demonstrate convergent roles for these genes in mid- and hindbrain morphogenesis (46, 48, 49). Coincident with morphogenesis, zebrafish develop reflexive, stereotyped escape behaviors that can be used to assess how developmental phenotypes affect functional neuronal output (50). Together the ability to identify convergent morphogenetic phenotypes and their impact on emerging neural circuits make zebrafish a well-suited model to study ASD gene function early in development.

Here we report that knockdown of SYNGAP1 and SHANK3 zebrafish orthologs produce common phenotypes associated with embryonic disruptions of brain morphogenesis. Consistent with functional roles that extend beyond synapses, a phylogenetic analysis of these proteins indicates that both SYNGAP1 and SHANK3 are vertebrate-specific versions of more ancient protein families that are found in animals lacking nervous systems. At the level of gene expression, zebrafish syngap1a, syngap1b, shank3a and shank3b are all expressed in the brain during embryogenesis. Knockdown of either syngap1b or shank3a
results in similar disruptions in the nervous system characterized by extensive neuronal cell death, pronounced developmental delay in mid- and hindbrain regions, and seizure-like behaviors. Moreover, co-injecting sub-phenotypic doses of syngap1b and shank3a morpholino (MO) recapitulates phenotypes seen in embryos injected with higher doses of either syngap1b or shank3a MOs, demonstrating a synergistic loss-of-function between syngap1b and shank3a. In summary, our data suggest that in addition to their roles at synapses, both shank3a and syngap1b are also required more broadly for brain morphogenesis.

RESULTS

SYNGAP1 and SHANK3 are duplicated in zebrafish

Zebrafish gene orthologs of human SYNGAP1 and SHANK3 were identified using Ensembl databases. While SYNGAP1 and SHANK3 each appear once in the human genome, both genes are duplicated in zebrafish (Fig. 1A,B). Using the Blast-Like Alignment Tool (BLAT; Kent informatics, Inc.), protein alignments of Homo sapiens and Danio rerio orthologs reveal a high level of amino acid identity: syngap1a (chromosome 19; 82.4%), syngap1b (chromosome 16; 84.9%), shank3a (chromosome 18; 84.5%) and shank3b (chromosome 4; 83.3%). A closer evaluation shows that all major functional domains in human SYNGAP1 are equally conserved in the two-zebrafish syngap1 ohnologs (Fig. 1A,B). By contrast, several important protein-protein interaction motifs contained in the proline rich and SAM C-terminal region, of human SHANK3 are more highly conserved in shank3a than shank3b. Therefore, shank3a is more suitable as a model for human SHANK3.
**SYNGAP1 and SHANK3 are innovations of the vertebrate lineage**

SYNGAP1 and SHANK3 are evolutionarily derived members of larger gene families that expanded by gene and whole genome duplication events in the vertebrate lineage (51). SYNGAP1, RASAL3, RASAL2, and DAB2IP belong to a sub-group of the Ras GTPase activating proteins (RasGAPs), distinguished from the larger RasGAP family by a typical domain structure: pleckstrin homology (PH), calcium dependent membrane localization (C2), Ras GTPase activating protein (RasGAP) and coil coiled domains (CC; Fig. 1C).

The SHANK1, SHANK2, and SHANK3 gene family is also distinguished by a typical domain structure: Ankyrin repeats (Ank), Src Homology 3 (SH3), Post-synaptic density protein 95/Disc large/Zona occludens 1 (PDZ), and Sterile Alpha Motif domains (SAM; Fig. 1D). Additional short Abp-, Homer- and Cortactin-interacting domains (52, 53) were previously identified in vertebrate Shanks 1-3 using functional assays and are not as clearly reflected by conserved sequence (Supplementary Material, Fig. S1). To identity unique signatures of vertebrate Shank3, multiple alignments of zebrafish, *Xenopus*, rat, mouse, and human Shank3 reveal 12 regions (each over 16 amino acids in length) of 80% or greater amino acid identity (Supplementary Material, Fig. S1), with only four of these twelve found in the single Shank protein encoded in the amphioxus genome. Like amphioxus, other metazoans have single versions of both a DAB2IP-like protein and a SHANK-like protein. Ancestral forms of both SYNGAP1 and SHANK can even be found in single-celled choanoflagellates. These proteins have the same collection of domains in a different order along the protein (Fig. 1C,D), indicating that SYNGAP1 and SHANK protein families originated before the divergence of single-celled animals and metazoans. In summary, both **SYNGAP1** and **SHANK3** are vertebrate innovations of
ancient proteins, suggesting a derived set of functional roles for these proteins in vertebrates.

**Zebrafish syngap1a/b and shank3a/b are expressed in the central nervous system during embryogenesis.**

To determine the developmental expression patterns of syngap1a, syngap1b, shank3a and shank3b, we harvested RNA from eleven developmental stages ranging in age from 2 to 120 hpf and used gene-specific primers for reverse transcription and quantitative polymerase chain reaction (RT-qPCR; Fig. 2A,B). Syngap1b, shank3a and shank3b transcripts are expressed from fertilization and gradually increase after 12-15 hpf, a developmental stage when the body-axis forms and the earliest neurons exit the cell cycle and start to differentiate (54). By contrast, syngap1a transcripts show constant expression until 36-48 hpf, when larval expression gradually increases. Together, these results show all four transcripts to be expressed in the early embryo.

To determine where syngap1a, syngap1b, shank3a and shank3b transcripts are expressed in zebrafish, we performed whole mount in situ hybridization at 48 hpf. All four transcripts are expressed in the brain with each showing unique patterns of concentrated expression (Fig. 2C,D). Syngap1a expression is most pronounced in the anterior forebrain and dorsal hindbrain. Like syngap1a, syngap1b is expressed in the dorsal hindbrain; however, syngap1b is also expressed at high levels in the optic tectum of the midbrain. Shank3a expression is most pronounced in the ventral regions of the fore-, mid- and hindbrain, while shank3b is expressed throughout fore- and midbrain with the highest levels of expression in the anterior-ventral forebrain. In addition to their expression in
the brain, syngap1b and shank3a also exhibit low levels of expression throughout the body consistent with their higher level of expression at early developmental stages by RT-qPCR (Fig. 2A,B).

**Splice-inhibiting morpholinos knockdown zebrafish syngap1 and shank3 ohnologs**

To functionally characterize the syngap1a, syngap1b, shank3a and shank3b genes, we designed splice-inhibiting morpholinos (MOs) against intron/exon junctions corresponding to conserved domains. To test gene knockdown efficacy, we amplified MO-targeted genes and a loading control elongation factor 1α (55) from cDNA generated from MO-injected larvae (Fig. 3A-C; Supplementary Material, Fig. S2). Syngap1a/b and shank3a/b MOs disrupted the expression of the targeted genes either by causing intron retention (syngap1a and shank3a/b) or by causing exon skipping (syngap1b). Based on sequencing of morphant-specific rtPCR products, all MOs would be predicted to result in N-terminal protein truncations (data not shown).

The syngap1a MO targets the exon 5/intron 5 splice-junction. Injection of syngap1a MO reduced syngap1a expression and produced three larger MO-specific bands due to intron retention (Supplementary Material, Fig. S2). The syngap1b MO targets the intron 3/exon 4 splice-junction and produced one smaller MO-specific band due to exon skipping (Fig. 3A). Both syngap1a and syngap1b MOs would be predicted to truncate the corresponding syngap1 protein in the pleckstrin homology domain.

The shank3a MO targets the exon 8/intron 8 splice-junction, produces intron retention, and would be predicted to truncate the protein within the fourth ankyrin repeat (Fig. 3B). The shank3b MO targets the exon 10/intron 10 splice-junction, produces intron retention
(Supplementary Material, Fig. S2), and would be predicted to truncate the protein within the sixth ankyrin repeat.

To test whether disrupting single shank3 and syngap1 ohnologs would impact expression of the others, we compared the levels of all four ohnologs in all morphant samples (Supplementary Material, Fig. S2). Consistent with its milder effect on phenotype, the shank3b MO specifically and completely disrupted expression of shank3b without impacting the expression of the other genes. By contrast, syngap1a, syngap1b, and shank3a MOs each reduced expression of all ohnologs compared to constant eef1a1/l levels. We attribute reduced syngap1a, syngap1b, shank3a and shank3b expression to a dramatic developmental delay in the nervous system produced by syngap1a, syngap1b and shank3a MOs (See sections on morphology and cell death).

**Dose/response curves identify doses and ohnologs for subsequent analyses**

By generating dose/response curves for syngap1a/b and shank3a/b MOs, we identified single doses that optimized the penetrance of gene-specific phenotypes without inducing general toxicity (Fig. 3D,E). These doses were then used for all subsequent phenotypic analyses. Dose/response analyses also identified syngap1b and shank3a MOs as the most penetrant at 48-72 hpf (Table 1; Fig. 3D,E) consistent with the dominant expression of these two ohnologs at early embryonic stages. Syngap1a and syngap1b were also co-injected to test for interplay between syngap1 ohnologs. Co-injection of 5 ng each of syngap1a/b MOs had an additive effect, suggesting syngap1a/b impact similar developmental processes (Fig. 3F). Despite this interplay between syngap1 ohnologs, singly injected syngap1a MO produced phenotypes of low penetrance and with
increasing doses became toxic. While not toxic at any dose, shank3b MO failed to produce a phenotype despite a molecularly efficient knockdown (Supplementary Material, Fig. S2). Due to a low phenotypic penetrance in syngap1a morphants and a lack of phenotypes in shank3b morphants, all subsequent analyses focus on knockdown phenotypes of syngap1b and shank3a ohnologs and the roles these genes play in the early embryo.

Due to a previously identified interaction between SYNGAP1 and SHANK3 in a yeast-2-hybrid screen (42), we tested whether syngap1b and shank3a function synergistically by co-injecting these MOs at doses (syngap1b MO (5 ng) or shank3a MO (4 ng)) that show low penetrance when injected singly. Syngap1b/shank3a double morphant phenotypes were comparable to single MO injections at higher doses and included altered mid/hindbrain boundaries, cardiac edema, developmental delay and unproductive swim bouts (Figs. 3G). To test whether this synergy between sygap1b and shank3a extended to other combinations of syngap/shank ohnologs, syngap1a/shank3a and syngap1b/shank3b were also co-injected (Table 1). These other co-injections did not produce morphological and behavioral phenotypes with the same penetrance, further supporting the specificity of synergy between syngap1b and shank3a.

Seizure-like behaviors and unproductive escape responses characterize both syngap1b and shank3a morphants

To quantify morphant behaviors, we conducted kinematic analyses of zebrafish escape responses at 72 hpf (56). Escape responses in larvae injected with control MO (CoMO) were highly stereotyped (Supplementary Material, Movie S1), characterized by a strong
C-bend away from the stimulus followed by sinusoidal undulations that decreased in frequency and amplitude as larvae swam away from the stimulus (Fig. 4A, CoMO). By contrast syngap1b (Supplementary Material, Movie S2), shank3a (Supplementary Material, Movie S3) and double syngap1b/shank3a (Supplementary Material, Movie S4) morphant swim attempts were unproductive, characterized by undulations with constant rather than graded frequencies and sustained amplitudes (Fig. 4A, syngap1b - 82%, shank3a - 86%, double syngap1b/shank3a - 75%). Indeed, rather than a transition from high to low amplitude bends as seen in CoMO larvae, syngap1b, shank3a and double syngap1b/shank3a morphants produced an inverted transition from low to high amplitude bends. These unproductive swim attempts were also characterized by significantly reduced swimming velocities for syngap1b (1.15±0.9 pixels/msec; n=8), shank3a (4.45±2.32 pixels/msec, n=8), and syngap1b/shank3a double morphants (1.8±1.23 pixels/msec, n=8), compared to control morphants (14.4±4.65 pixels/msec, n=8; Fig. 4B). In addition to reduced swimming velocities, syngap1b morphants also displayed significantly increased swimming-bout durations (858±552 msec; n=20) compared to control morphants (247±169 msec; n=15; Fig. 4C). In addition to increased swimming bout duration similar to syngap1b morphants, shank3a morphants also exhibited a single coiled response, recapitulating the main phenotype reported in a previous study. (57). In contrast to the previous study, we did not observe single coiled responses in shank3b morphants. In our experiments, both syngap1b and shank3a morphant behavioral phenotypes were most pronounced between 48 and 72 hpf.

A subset of behaviorally phenotypic syngap1b (18%; Supplementary Material, Movie S5), shank3a (30%; Supplementary Material, Movie S6), and double syngap1b/shank3a (8%;
Supplementary Material, Movie S7) morphants also exhibited spontaneous (occurring in the absence of a touch stimulus) seizure-like behaviors characterized by prolonged (lasting seconds to minutes rather than the typical <500 msec) unproductive swim bouts (Fig. 3D,E&G), suggesting that knock-down of either syngap1b, shank3a or the combination produces hyper-excitability in the nervous system.

Reducing syngap1b or shank3a and double syngap1b/shank3a alters brain morphology and increases cell death in embryonic nervous system

In addition to behavioral deficits, syngap1b, shank3a, and double syngap1b/shank3a morphants exhibited altered brain morphologies suggestive of developmental delay (Fig. 5). At 28-30 hpf syngap1b, shank3a, and double syngap1b/shank3a morphants had malformed mid/hindbrains (Fig. 5A,B). At 48-52 hpf syngap1b, shank3a, and double syngap1b/shank3a morphant heads were still curved around the yolk as would be stage-appropriate for 24-28 hpf (Fig. 5A,C) and some larvae had edema around their hearts (Fig. 5C,D). At 72 hpf, syngap1b, shank3a, and double syngap1b/shank3a morphant developmental delay was still apparent with head morphologies resembling CoMO injected larvae at 48 hpf. In addition to developmental delay, shank3a morphants had shorter tails. These qualitative changes in gross morphology indicate syngap1b, shank3a, and double syngap1b/shank3a morphants have delayed brain development.

To better understand hyper-excitable phenotypes in 48 hpf syngap1b, shank3a and double syngap1b/shank3a morphants, we quantified excitatory (glutamate-using stable vglut transgenic) and inhibitory (GABA-using antibody staining) neurotransmitter expression in transverse frozen brain sections. This approach also enabled us to compare cross-
sectional areas for 48 hpf fore-, mid- and hindbrain sections of *syngap1b*, *shank3a* and CoMO larvae (Supplementary Material, Table S11). Overall, *syngap1b* and *shank3a* morphant brains were microencephalic as reflected in significantly decreased cross-sectional areas in all brain regions measured. Moreover, reductions in the mid- and hindbrain areas could largely be accounted for by the absence of dorsal structures in both *syngap1b* and *shank3a* morphants (Fig. 6A). Both *syngap1b* and *shank3a* morphants were characterized by a significant reduction of GABAergic neurons in the midbrain and hindbrain, while vglut expression was only significantly reduced in the hindbrain (Fig. 6B,C, Table 2,3). These results demonstrate the potential for hyper-excitability in the midbrain of *syngap1b* and *shank3a* morphants. Developmental delay was improved but still apparent in 96 hpf *syngap1b*, *shank3a* and double *syngap1b/shank3a* morphant larvae, wherein dorsal structures had formed but overall morphology remained delayed in mid/hindbrain regions with 96 hpf *syngap1b* and *shank3a* morphant brains resembling those of controls at 48 hpf (Supplementary Material, Fig. S3).

By contrast to delays in the mid/hindbrain regions, *syngap1b* and *shank3a* morphant spinal motor neurons were stage-appropriate (Fig. 7). Spinal motor neurons were visualized using SaigFF213A that drives GFP in Caudal Primary motor neurons, a subset of interneurons and Rohon Beard sensory neurons (58). These transgenic morphant embryos were then injected with tetramethylrhodamine-Bungarotoxin, to visualize post-synaptic clusters of acetylcholine receptors (59). Co-localization of motor axons with receptors reflects that morphant motor neurons have formed synapses on their post-synaptic muscle targets (Fig. 7). In addition to demonstrating that spinal motor neurons were not delayed in *syngap1b* and *shank3a* morphants, this analysis stickingly revealed
that Rohon Beard neurons were reduced/absent in \textit{shank3a} but not \textit{syngap1b} morphants (Fig. 7B,C).

To determine whether developmental delays in brain development might be explained by earlier developmental disruptions, we quantified cell death by staining one-day-old morphant embryos with acridine orange (60). \textit{Syngap1b}, \textit{shank3a}, and double \textit{syngap1b/shank3a} morphants all exhibited increased cell death in the central nervous system (CNS) that was most pronounced at 24-28 hpf. In both \textit{syngap1b} and \textit{shank3a} and double \textit{syngap1b/shank3a} morphants, cell death was pronounced in the midbrain, hindbrain, and spinal cord (Fig. 8A,B). Because cell death is a common off-target result of siRNA and morpholino knockdown technologies (61), morphants were coinjected with either orthologous human mRNA or \textit{p53} MO to distinguish between gene-specific and off-targeting effects, respectively. In \textit{syngap1b} morphants, co-injection of the \textit{p53} morpholino did not rescue cell death (Fig. 8A, n=3). By contrast, in \textit{shank3a} morphants, co-injection of the \textit{p53} MO almost entirely rescued cell death (Fig. 8B, n=3). Although cell death rescue with \textit{p53} MO can suggest off-targeting effects, \textit{SHANK3} mRNA co-injection also rescued cell death indicating that cell death is gene-specific and that decreasing \textit{shank3a} expression may trigger one of several \textit{p53}-dependent cell death pathways (62).

Indeed, cell death could be partially rescued for both \textit{syngap1b} and \textit{shank3a} morphants by injecting the longest isoform of human \textit{SYNGAP1} and \textit{SHANK3} mRNAs respectively, suggesting that morphant cell death was gene-specific (Fig. 8C,D). One-way ANOVAs with \textit{post hoc} means comparisons (Tables 1,2, Supplementary Material, Tables S7,8) revealed that cell death was significantly increased in all brain regions for both \textit{syngap1b}
and shank3a knockdown experiments (Supplementary Material, Tables S9.1-10.3). Co-injection of syngap1b MO and human SYNGAP1 mRNA produced a nearly complete rescue, significantly reducing cell death in the hindbrain and spinal cord compared to embryos injected with syngap1b MO alone (Supplementary Material, Tables S9.2, 9.3). Likewise, co-injection of shank3a MO and human SHANK3 mRNA produced a partial rescue, significantly reducing cell death in the midbrain, hindbrain and spinal cord, compared to embryos injected with shank3a MO alone (Supplementary Material, Table S10.1-10.3). Overall, these results indicate that decreasing expression of either syngap1b or shank3a causes increased CNS cell death.

Similar to singly injected morphants, syngap1b/shank3a double morphants exhibited increased cell death in the midbrain and hindbrain compared to controls (Supplementary material, Table S9.1-10.3). However, in comparison to singly injected morphants, syngap1b/shank3a double morphants did not show significantly increased cell death of the spinal cord. This brain-specific increase of cell death in syngap1b/shank3a double morphants suggests that disruptions in the mid- and hindbrain are sufficient to cause morphant behavioral phenotypes.

**DISCUSSION**

Our results demonstrate that prior to their established roles in synaptogenesis, SYNGAP1 and SHANK3 play critical roles in embryonic development. A major research focus in models of human SYNGAP1 and SHANK3 haploinsufficiency has been synapse function (24, 28, 32, 36-39, 44, 63-67). While reductions in SYNGAP1 or SHANK3 gene expression induce overlapping behavioral phenotypes in both animal models and
individuals with ASD (36-38, 41, 68), these proteins have divergent roles at
 glutamatergic synapses: reductions in SYNGAP1 precociously strengthen synaptic
 transmission (37, 43) while reductions in SHANK3 weaken synaptic transmission (38, 69). Therefore, to look beyond their defined synaptic role and to complement previous mammalian models, we chose zebrafish to provide an embryonic window into the unknown developmental roles of SYNGAP1 and SHANK3.

By assessing developmental phenotypes in zebrafish morphant models of SYNGAP1 and SHANK3 we build upon studies modeling ASD-linked genes DISC1, CHD8, MET, AUTS2, FMR1 and SHANK3 in zebrafish (46, 48, 57, 70-72). In addition to embryonic phenotypes that are consistent with previous zebrafish ASD models, we describe a digenic model wherein a combined sub-phenotypic reduction in gene expression of both syngap1b and shank3a recapitulate phenotypes seen in more severe single gene loss-of-function mutations. Therefore, our study is unique in directly comparing two ‘single gene’ ASD models, demonstrating their genetic synergy, and bringing to light both commonalities and differences in underlying embryonic processes that explain phenotypes. We find that reducing either single gene or a mild reduction of both genes resulted in embryonic cell death, developmental delays, unproductive locomotion and seizure-like behaviors. We suggest that this early pathology may contribute to later phenotypes by disrupting embryonic critical periods essential for mature neural circuit function (73).


**SHANK3 and SYNGAP1 are expressed at embryonic stages in the central nervous system.**

As we report here for zebrafish, SYNGAP1 and SHANK3 orthologs are also expressed at embryonic stages in the nervous systems of human, mouse and Xenopus (74-78). In humans at 8 weeks post-conception SYNGAP1 expression is most pronounced in the developing hippocampal formation and amygdaloid complex, while both SYNGAP1 and SHANK3 show pronounced expression in the nascent cerebellum (upper rhombic lip), (77). In mouse embryos, Syngap1 is expressed broadly throughout the body (E8.5) with a progressive restriction to the brain (E10.5) and then to specific brain regions (E16.5), such as the hippocampus, hypothalamus, dorsal thalamus and basal ganglia (74). Mouse Shank3 expression has been reported in cardiac progenitor cells (E7.5-8.5) and later in whole brain cortex (E15; (75, 76). Additionally, *Xenopus laevis* frog embryos express shank3 during embryogenesis in the cardiovascular system, isthmus, brain, neural tube, retina and hypochord (78). Taken together, both SYNGAP1 and SHANK3 are expressed broadly in vertebrate embryos with a progressive enrichment to brain regions during development. Despite these expression profiles, the developmental roles of Syngap1 and Shank3 prior to synaptogenesis remain unknown.

**Syngap1b and shank3a play critical roles in brain morphogenesis**

Consistent with embryonic SYNGAP1 and SHANK3 expression in vertebrates, knockdown of syngap1b, shank3a, or the combination disrupts brain morphogenesis suggesting novel embryonic roles for these genes. Our findings are in line with a recent and growing body of literature indicating that ASD pathobiology starts in the early
embryo. For example, zebrafish knockdown and mutant models of \textit{zDISC1} and \textit{chd8} have revealed novel roles for these genes in regulating brain morphology as early as gastrulation (48, 49). In addition, \textit{DISC1} and ANK3 mutant mouse models have functionally linked these genes to neuronal migration during embryonic brain morphogenesis (79-81). Several studies on human ASD post-mortem tissues have suggested that neural patterning processes in the embryo may contribute generally to ASD pathophysiology (82-84). The disorganized cytoarchitecture and cortical layering typical of post-mortem ASD brains may be explained by aberrant neurogenesis and cellular migration in the embryo (83, 85). Therefore, our findings are consistent with an emerging viewpoint that ASD pathobiology starts at embryonic stages when diverse signaling pathways regulate proliferation, migration, and cell death of neural progenitors to shape the brain.

Likely a consequence of altered brain morphogenesis, another common phenotype in individuals with ASD is developmental delay. Indeed, developmental delays are a consistent diagnosis in patients with \textit{SHANK3} and \textit{SYNGAP1} mutations; however, the underlying causes of these developmental delays are largely unknown (23, 24, 28, 31, 63). Our results indicate that commonalities observed in developmental delays in zebrafish \textit{syngap1b} and \textit{shank3a} morphants arise though distinct mechanisms. Despite the fact that both \textit{syngap1b} and \textit{shank3a} models show increased cell death, the underlying mechanisms for cell death differ in \textit{syngap1b} and \textit{shank3a} morphants, with only \textit{shank3a} morphant cell death occurring through a \textit{p53}-dependent pathway. Despite the ability to rescue \textit{syngap1b} and \textit{shank3a} morphant cell death with mRNAs encoding human orthologs, we are tentative about the extent of gene specific cell death because cell death
is also a commonly reported phenotype associated with morpholino toxicity. With this in mind, our results suggest that distinct molecular pathways underlie cell death in *syngap1b* and *shank3a* morphants yielding phenotypically similar developmental delays.

**Behavioral phenotypes suggest disruptions in hindbrain regulation of swim circuits**

Swimming behaviors are a sensitive indicator of early nervous system function in zebrafish due to a highly stereotyped and rapidly expanding repertoire of locomotor behaviors produced by well-characterized neuronal circuits (86, 87). Early behaviors are produced by some of the first neurons to be born during neurogenesis. These include descending mid- and hindbrain interneurons that drive rhythmic firing in motor neurons to produce one of the first coordinated locomotory behaviors, the escape response (88). *Syngap1a, syngap1b, shank3a* and double *syngap1b/shank3a* morphant escape responses are characterized by unusually long-lasting and unproductive swimming which could result from reductions in the number of descending brain neurons that would normally function to activate and inhibit spinal cord circuits. This idea is supported by work in both zebrafish, where swimming behavior is triggered via hindbrain descending Glutamatergic V2a neurons, and *Xenopus laevis*, where neuronal activity in mid/hindbrain GABAergic neurons is associated with terminating bouts of swimming (89-91). Our data suggest that a stage-appropriate neuromuscular system paired with significantly delayed mid- and hindbrain regions could account for *syngap1* and *shank3* morphant behavioral deficits.

In addition to weaker swims, a proportion of *syngap1b, shank3a* and double *syngap1b/shank3a* morphants exhibited seizure-like behaviors consistent with some level
of hyper-excitability or lack of proper inhibition. A disruption in establishing or maintaining the balance between neurons expressing excitatory and inhibitory neurotransmitters (E/I balance) has been put forward as a major convergent phenotype in individuals with ASD (8, 73, 92). Morphological analyses of postmortem brains from individuals with ASD find disordered GABAergic and glutamatergic processes in cortical mini-columns and depressed production of inhibitory GABA (93, 94). E/I balance has also been explored in animal ASD models: Gogolla and colleagues conducted a meta-analysis of ASD mouse models and found that a reduction in GABAergic neurons was a common phenotype with functional implications for early critical periods (73). The importance of E/I balance is underscored by the prevalence (25-30%) of epileptic symptoms in individuals with ASD, including those carrying SYNGAP1 and SHANK3 mutations (22-24). Epilepsy has been thought of as a disruption in E/I balance generating periods of asynchronous and abnormal behavior (92). Our results suggest that hyper-excitable behaviors are linked to brain-region-specific developmental delays. This hypothesis bears testing in the context of known circuits in stable mutant models.

**Functional interactions and network susceptibility may explain syngap1b and shank3a phenotypes**

Individuals with ASD tend to carry a higher mutational burden for both SNPs and CNVs (95). Although mutations linked to ASD occur in hundreds of distinct genes, common genetic and protein networks indicate high functional connectivity among genes and proteins causal for ASD. This connectivity is observed between Syngap1 and Shank3 that have been suggested to interact directly, while being functionally connected within the post-synaptic density of glutamatergic neurons (42). In addition, these functionally
connected gene clusters, such as those associated with synapses, tend to have overlapping temporal and spatial gene expression (96). These patterns of expression indicate crucial networks, that when disrupted, bias an individual towards ASD. Therefore it is not surprising that single gene knockdowns, or a combination of syngap1b and shank3a dual knockdowns produce similar phenotypes in light of their shared functions, protein interactions and temporal and spatial expression patterns.

CONCLUSION PARAGRAPH

Here we report for the first time that syngap1 and shank3 serve important functional roles at early stages of vertebrate brain morphogenesis that precede synaptogenesis. These models demonstrate that knocking down syngap1b and shank3a causes CNS-specific cell death and developmental delay in mid- and hindbrain regions that likely contributes to hyper-excitable behavior. By providing this window into early developmental, our findings in zebrafish support the emerging viewpoint that ASD gene mutations likely impact neonatal stages contributing to ASD pathology. In the future, it will be critical to establish stable zebrafish mutants to investigate the shared causes and long-term functional consequences of early developmental dysfunction.

MATERIALS AND METHODS

Fish maintenance and embryo rearing

Transgenic lines Tg vglut2:dsRed (97) and Tg SaigFF213A (82) were used for all morpholino knockdown experiments. After briefly rinsing zygotes in deionized water, embryos and larvae were raised in glass petri plates filled with 28°C “system water” (water from aquaria housing adult zebrafish) and staged by hours post fertilization (hpf).
Both adults and embryos were maintained on a 14:10 circadian light:dark cycle. Fish in our facility are cared for in accordance with National Institute of Health (NIH) animal care guidelines and the University of Miami’s Institutional Animal Care and Use Committee (IACUC) have approved all animal protocols.

**Evolutionary comparison of Syngap1 and Shank proteins**

Sequences of proteins belonging to the DABIP2 (Rasgaps including Syngap1) and Shank families were identified in genomes by using the NCBI blast program and confirmed by best reciprocal blast. Presence or absence data were collated and mapped onto a phylogenetic tree of animals (98). For the analysis of Shank3, sequences were aligned using Clustal Omega (99) and imported into Adobe Illustrator (Adobe Systems Inc. San Jose, CA) where regions of high local similarity were identified.

**Whole mount in situ hybridization**

Larvae were dechorionated and placed in a mixture of fish facility system water and 0.003 % phenothiourea at 24 hpf. RNA syntheses of sense and anti-sense probes were carried out using the DIG RNA labeling kit (SP6; Roche, Mannheim, Germany). See Supplementary Material, Table S1 for in situ hybridization primers used to amplify and synthesize RNA probes. The in situ protocol was adapted from a previous study, with a hybridization buffer containing 70% formamide and incubations carried out at 55 °C (100). Whole mount images were captured using an Olympus D71 micro-imager mounted on a compound Olympus microscope at 40x and 100x magnification.
RT-qPCR developmental time course

Total RNA was extracted from zebrafish embryos at 2, 5, 8, 12, 15, 24, 36, 48, 72, 96, and 120 hpf using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Three samples consisting of 25-pooled embryos at each time point were used for RNA extraction. Extracted RNA was treated with DNase (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. All RNAs and templates were quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Pittsburgh PA, USA), with 260/280 ratios of between 1.9-2.0. Whole RNA samples were run on 1% agarose gels to determine RNA integrity. RT-qPCR was performed using the qScript One-Step SYBR Green qRT-PCR kit (Quanta Biosciences, Gaithersburg, MD, USA) to test the expression level of syngap1a, syngap1b, shank3a, shank3b and eef1a1/1 during development. eef1a1/1 was chosen as a housekeeping gene in a previous study (101) due to its consistent expression profile following 5 hpf. Primers (Integrated DNA Technologies, Coralville, IA, USA) for each gene used in this study were designed using primer3 and BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat) against the zebrafish genome to confirm gene specificity (102). Each RT-qPCR reaction contained: 1X One-Step SYBR Green Master Mix, 200 nM forward and reverse gene specific primer, 100 ng RNA template, 1X qScript One-Step RT and nuclease-free water to a final volume of 50 µL. The cycling conditions were: 48°C for 10 min; 95°C for 5 min; 40 cycles of 95°C for 10s, 60°C for 20s, and 72°C for 45s; 72°C – 95°C for 20 min (melt analysis); 95°C for 15s. RT-qPCR reactions were run using an Eppendorf Mastercycler ep realplex (Eppendorf, Hamburg, Germany). Absolute quantification of each sample was based on
linear regression of standard curve samples (See Supplementary Material Methods and Supplementary Material, Tables S2-5 for RT-qPCR validation and analysis.)

**Antisense morpholino oligonucleotide injections**

Splice-inhibiting morpholino oligonucleotides (MOs; Gene Tools, LLC. Philomath, OR, USA; Supplementary Material, Table S6) were designed against the common exon-intron boundaries based upon *syngap1a, syngap1b, shank3a* and *shank3b* variants annotated in the Ensembl database. We found that the genome annotation of *shank3a* was inaccurate with the single gene reported as two discontinuous 3' and 5' segments. We designed a forward (5'-TGGAAACTTTGACCTGGCAG-3') and a reverse primer (5'-GGGAGTGTGAGGAC AACGAG-3') that spanned the gap and sequenced this middle portion of the *shank3a* gene. MO design was based on this assembled sequence and the correction was reported to Ensembl (GenBank accession no. LM994718). Lyophilized MOs were resuspended in nuclease free H₂O as 1 mM stock solution, aliquotted, and stored at 4°C. To ensure full resuspension of stored MOs, stock solutions were heated to 65°C prior to making dilutions for injections. For injections, MOs were diluted with 1% fast green and injected into one-cell zygotes. To ensure that only successfully injected embryos were further analyzed, a few hours after injection, embryos were sorted for even distribution of the green color throughout yolk and embryo. For each morpholino, we generated dose-response curves by injecting a series of dilutions (2nL bolus at 1, 0.5, 0.25 mM), scoring resulting phenotypes, and comparing these to embryos injected with molarity-matched standard control morpholino (CoMO, Gene Tools, LLC). Dilutions with the highest behavioral penetrance that lacked severe morphological defects were used for more detailed analyses. To explore the possibility of synergy between gene
duplicates (ohnologs), MOs for syngap1a and syngap1b were co-injected at doses that
failed to cause with phenotypes when singly injected. The MOs from syngap1b and
shank3a, syngap1a and shank3a, and syngap1b and shank3b were also co-injected at sub-
phenotypic doses to test for synergistic effects between zebrafish SYNGAP1 and
SHANK3 orthologs at the level of behavior and morphology.

**RT-PCR and sequencing to validate MO knockdown**

RNA was harvested from a time series of 24, 32, 48, and 72 hpf embryos and larvae
using TRIzol followed by DNASE treatment according to standard protocols (Life
Technologies, Carlsbad, CA, USA). For cDNA synthesis, 1 μg of RNA was reverse
transcribed using the SuperScript III™ First-Strand Synthesis System (Life
Technologies). cDNA was then used as template for PCR to test for mis-splicing events
induced by the MOs. Primer pairs for sequencing spanned each exon/intron boundary
targeted by morpholinos (See Supplemental Material, Table S3 for a list of these
primers.) However, the wild-type PCR product for shank3a did not show a shift in band
size or number of bands, therefore we elected to use an intron reverse primer to show
intron retention. Each PCR reaction contained: 1X GoTAQ hot start green master mix
(Promega, Madison, WI), 500 nM forward and reverse primer, 100 ng of cDNA template
and nuclease-free H2O to a final volume of 10 μL. The cycling conditions were: 95°C for
2 min; 35 cycles of 95°C for 30s, 55°C for 30s, 72°C for 1 min; 72°C for 5 min and 4°C
for storage. PCR products were run on a 1% agarose gel and checked for banding patterns
and relative band intensities. Bands were gel-purified, and re-amplified for sequencing to
determine how splicing was impacted by MO injections. To document morphant
morphological defects, pictures were captured on an AxioCam MRm (Zeiss, Inc., Jena, Germany).

**Behavioral and morphological analysis**

To analyze morpholino-induced behavioral phenotypes, high-speed videos were captured using a Fastcam 1024PCI (Photron USA Inc., San Diego, CA, USA). This camera was either mounted on a dissecting scope, or for FLOTE analysis, mounted with a Fujinon lens in a customized behavioral chamber. Parameters were as follows: shutter speed of 1/1000 using an LED array for backlit illumination (Advance Illumination; Rochester VT; Backlight LED Illuminator), 512 x 512 resolution, a frame rate of 250 f/s to assess swimming qualitatively and 1000 f/s to assess swimming kinematics. Three touch-evoked behaviors, elicited using tungsten or fishing line probes, were recorded per individual. After capturing videos, Flote (56) was used to quantitate changes in axis curvature over time and swimming velocity. Shadows from the tungsten probe disrupt the Flote tracking software, and were lessened using fishing line probes.

For morphological analysis, images were captured on an Olympus D71 advanced micro-imager mounted on a Wild dissecting microscope. For cell death rescue experiments, the human *SYNGAP1* mRNA construct was cloned into a pCS2+ backbone, while the human *SHANK3* α-isoform was codon optimized using GeneART (Life Technologies) to address cloning difficulties associated with high GC content. Human *SYNGAP1* and *SHANK3* were linearized with SacII and NotI, respectively and capped mRNA was transcribed using the SP6 mMessage mMachine kit (Life Technologies). Acridine Orange (Sigma, St. Louis MO) was used to stain for cell death following a previously published protocol (60).
After staining, embryos were anesthetized using 0.02% MS222 and mounted on a glass bottom dish in 3% methylcellulose. Images of stained embryos were collected as z-stacks on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

**Excitatory/Inhibitory Immunohistochemical staining**

To measure excitatory and inhibitory neurons, vglut2:DsRed embryos were counter stained with a primary antibody targeting γ-aminobutyric acid (GABA, a polyclonal GABA anti-rabbit, Sigma Aldrich, Poole, England). Morphants were collected and sorted at 48 hpf for vglut2:DsRed fluorescence and characteristic swimming behaviors. Prior to fixation, larvae were anesthetized on ice for 30 minutes. The water was then replaced with 4% paraformaldehyde (16% stock, Pierce Protein Biology Products, Rockford, IL, USA) in 1x phosphate buffer solution (PBS) and samples were incubated on a rocker for two hours at room temperature. After three washes of 1x PBS, samples were placed in a 1x PBS, 30% sucrose solution and rocked at 4°C for two hours. Larvae were then embedded in tissue-freezing medium (Triangle Biomedical Sciences, Inc., Durham, NC, USA) and orientated to achieve horizontal section of the forebrain and transverse sections of the mid- and hindbrain. Frozen blocks of tissue were sectioned on a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). Slides were incubated at room temperature for 10 minutes and then transferred to a Sequenza™ Slide Rack in Coverplates™ (Thermo Shandon Limited, Astmoor, Runcorn Cheshire WA7 1PR) and washed with a 0.4% TritonX-100 PBS solution (PBSTx). After washing, the sections were incubated in blocking solution (5% donkey serum in PBSTx) for one hour followed by an overnight incubation in primary antibody PBSTx solution. A primary antibody targeting GABA was used at concentrations of 1:500 and 1:1000 for 48 hpf and 96 hpf,
respectively. Sections were then washed six times with 1x PBS, followed by a two-hour incubation in a fluorescent secondary antibody (Alexa Fluor, goat anti-rabbit IgG, Molecular Probes, Eugene, OR, USA). Following secondary incubation, the sections were again washed six times with 1x PBS. Sections were then mounted using 1-thiodiethanol (Sigma, St. Louis MO) and viewed on a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). Single frames and z-stacks were captured for the 48 and 96 hpf sections respectively.

Quantification of dye and immunohistochemical staining

Analyses of acridine orange staining and vglut2:dsred/GABA expression were performed using Fiji (103). For acridine orange staining of brain regions, sagittal z-stacks were measured using the percent area fraction function with an IsoData automatic threshold. The percent of acridine orange staining was then compared via ANOVA, with a post-hoc students t-test means comparison using Bonferroni correction for multiple comparisons in the statistical package JMP (SAS institute inc.). Fiji was also used to measure the area of immunohistochemical staining in transverse sections from forebrain (anterior-mid thalamus and anterior optic tectum), posterior forebrain/midbrain (hypothalamus to tegmentum) and hindbrain (medulla oblongata). Differences in morphant GABA expression were quantified by counting GABA/DAPI positive cell bodies in brain hemisegments. In contrast to GABA, vglut expression was quantified using the Fiji area fraction function because we could not distinguish individual cell bodies in the dorsal-most brain regions. The overall cortical area of specific brain regions was recorded to assess differences in overall brain size. Area measurements were calculated in microns.
squared. Statistical analyses were performed using Students t-test. Brain regions were annotated following the scheme of Mueller et al. 2006 (104).

SUPPLEMENTAL MATERIAL

Supplemental material is available at HMG online.

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REFERENCES


associated with adult hippocampus-specific SynGAP1 knockout. Learn. Mem., 19, 268-281.


**FIGURE LEGENDS**

**Figure 1.** Protein diagrams and phylogenetic trees depict domain order and gene expansion through evolutionary time. (A&B) Protein diagrams of *SYNGAP1* and *SHANK3* orthologs for *H. sapiens* and *D. rerio* were generated using SMART (http://smart.embl-heidelberg.de/) from published Ensembl protein sequences. Diagrams represent the longest isoform available for each ortholog, denoted by species name and chromosome number (chr.). All major functional domains are conserved between the *H. sapiens* and *D. rerio* gene duplicates with the exception that *shank3b* has a sixth ankyrin repeat. Scale bar = 200 amino acids (aa). *SYNGAP1* protein domains: PH - plekstin homology, C2 - calcium dependent membrane localization, RasGAP - GTPase activator protein and CC - coiled coil. *SHANK3* protein domains: ANK - ankyrin repeat, SH3 - SRC homology 3, PDZ - Post Synaptic Density-Drosophila disc large suppressor-Zonula occludens-1, SAM - sterile alpha motif. RasGAP sub-family (C) and Shank family (D) proteins from diverse animal phyla were identified by best reciprocal blast with Human *SYNGAP1* and *SHANK3* and are mapped onto a phylogenetic tree adapted from a recent genomic study (69). This analysis highlights deep evolutionary roots of both *SYNGAP1* and *SHANK3* protein families with expansion by genome duplication events (*) in vertebrates and again in teleost fish. Within expanded vertebrate gene families, *syngap1*
and \textit{shank3} are derived from ancestral family members most similar to \textit{DAB2IP} and \textit{Shank2}.

\textbf{Figure 2.} Zebrasfish \textit{shank3} and \textit{syngap1} ohnologs are expressed during embryogenesis and enriched in the brain. Developmental gene expression of (A) \textit{syngap1a} and \textit{syngap1b} and (B) \textit{shank3a} and \textit{shank3b} from 2 to 120 hours post fertilization was determined using RT-qPCR. Absolute copy numbers were derived for each gene and plotted as a log function, adjusted for no template control, with error bars denoting standard deviation for three RNA sample replicates per developmental time-point. The asterisk for \textit{syngap1a}-48 hpf represents an outlier by three orders of magnitude. 48 hpf whole mount \textit{in situ} hybridization gene expression patterns for (C) whole body and (D) brain and rostral spinal cord. \textit{Syngap1a} is enriched in the dorsal hindbrain and anterior forebrain, while \textit{syngap1b} is enriched throughout the midbrain and hindbrain. \textit{Shank3a} is enriched throughout the entire brain, while \textit{shank3b} is enriched in the anterior forebrain. Scale bars = 500 \textmu m (C) and 250 \textmu m (D). The line drawing of the brain was adapted from Mueller et al. 2006 with fore-, mid-, and hindbrain regions denoted by shades of grey.

\textbf{Figure 3.} Splice-inhibiting morpholinos target intron/exon junctions resulting in dosage sensitive phenotypes at 48 hpf. Morpholino design strategy and RT-PCR detection of splice-inhibiting events for \textit{syngap1b} (A), \textit{shank3a} (B) and \textit{syngap1b/shank3a} (C) morphants. Arrows and dashed lines represent PCR primers and amplicons, respectively. Exons (boxes) and introns (bent lines) are not drawn to scale. Morphant cDNA used as template for PCR are noted above each gel while genes targeted by primer sets are indicated to the right. Morphant templates are abbreviated as \textit{syng1b} and \textit{shk3a} for \textit{syngap1b} and \textit{shank3a}, respectively. (D-F) Stacked histograms show percentages of
larvae exhibiting mortality and unproductive swim bouts (proportion with seizure-like behavior shown by hatching) with different amounts of morpholino injected. Morphants that displayed severe morphological phenotypes such as curved and bent tails were not assayed for swimming ability. Injection amounts producing the highest behavioral penetrance of 10 and 8 ng were used for (D) syngap1b MO (n=131) and (E) shank3a MO (n=125), respectively. Co-injection of either (F) syngap1a/syngap1b (5/5 ng, n=16) or (G) syngap1b/shank3a MO (5/4 ng, n=121) recapitulated phenotypes observed in higher doses of singly injected morpholinos. CoMO injected fish showed no defects in swimming behavior on days assayed (n=87).

**Figure 4.** Escape responses in 72 hpf syngap1b, shank3a and syngap1b/shank3a morphants are unproductive. (A) Touch-evoked escape responses were recorded using a high-speed camera and analyzed using FLOTE analysis software. FLOTE software divides the larvae into three segments and swimming traces depict body curvature as the sum the angles between segments, where positive values denote a bend in one direction and negative values bends in the opposite direction. Syngap1b, shank3a and syngap1b/shank3a double morphants show a steady swimming frequency, reduced flexion and increased swimming durations in comparison to controls. (B) Swimming velocities (pixels/msec) were determined using the kinematic analysis function in FLOTE. (C) Bout lengths were calculated in milliseconds as the first to last frame of a continuous swimming bout. Both swimming velocity and bout lengths were analyzed by students t-test between syngap1b, shank3a and syngap1b/shank3a morphants and CoMO. Box plot lines represent the first quartile (bottom), median (middle) and third quartile (top). Asterisks indicate significance, * = p<0.01 (Supplementary Material, Table S6).
**Figure 5.** Brains of *syngap1b* and *shank3a* morphants at 28-30, 48 and 72 hpf show developmental delay compared to control morphants. At 28-30 hpf, both *syngap1b* and *shank3a* morphants have changes in ventricle size and disruptions of the mid-hindbrain boundary (white arrowheads). At 48 and 72 hpf both *syngap1b* and *shank3a* morphants exhibit microencephaly and developmental delay with curved head to trunk angles (curved arrow) that resemble earlier control stages. Both *syngap1b* and *shank3a* morphants also sometimes have cardiac edema (black arrowheads) and *shank3a* morphants have a shorter body axis. Scale bars = 1 mm (28-30, 48, 72 hpf) and 250 μm (28-30 hpf).

**Figure 6.** Excitatory (vGluT) and inhibitory (GABA) neurons are reduced in 48 hpf *syngap1b* and *shank3a* morphants. Three transverse sections represent the three major brain divisions; forebrain, midbrain and hindbrain. Scale bars = 50 μm. The line drawn brain atlas was adapted from Mueller et al. 2006. Brain regions are indicated in wild type sections: DTh - dorsal thalamus, H - hypothalamus, MO - medulla oblongata, T - tegmentum, TeO - Optic Tectum, and VTh - ventral thalamus. Expression of vGlut and GABA in *syngap1b*, *shank3a* and control morphant (D) midbrain and (E) hindbrain regions were quantified in Fiji as a percentage of brain area. Size on the x-axis is defined as the percentage of total brain area (μm²). All values were normalized to CoMO and analyzed using students t-test. Asterisks indicate significance values, * - p<0.05, ** - p<0.01, *** - P<0.001, **** - p<0.0001 (Table 3-4).

**Figure 7.** Motor neuron innervation of muscle is stage-appropriate in 48 hpf *syngap1b* and *shank3a* morphants. Lateral views of morphant spinal cords in stable transgenic SAIGFF213A:GFP embryos injected with Tetramethylrhodamine α-Bungarotoxin to
label acetyl choline receptors (AchRs). In the SAIGFF213A:GFP line, GFP is expressed in Caudal Primary (CaP) motor neurons and Rohon-Beard (Rb) sensory neurons. 

*syngap1b* (n=4), *shank3a* (n=4) and control (n=4) morphants all exhibit stage-appropriate CaP motor neurons (green) and AchR clusters in muscle (red). Rb neurons are absent in *shank3a* morphants but present in both control and *syngap1b* morphants. Scale bars = 25 μm. The diagram in the lower right serves as a key.

**Figure 8.** Cell death is increased in 28-30 hpf *syngap1b, shank3a* and *syngap1b/shank3a* morphants. (A) *shank3a* MO and (B) *syngap1b* MO were stained with acridine orange (AO) to visualize cell death. Morphants were also co-injected either with the corresponding human mRNA or *p53* MO to assess the nature of the cell death pathway and whether cell death was gene-specific. Scale bars = 100 μm. (C-D) Staining was quantified using the percent area fraction method (Fiji) in midbrain, hindbrain, and spinal cord regions. Death is partially rescued in hindbrain and spinal cord regions by co-injection of either human (C) *SYNGAP1* mRNA (n=10) or (D) human *SHANK3* mRNA(n=10), respectively. Data were analyzed using a one-way ANOVA with a post-hoc means comparison and Bonferroni correction. Box plot lines represent the first quartile (bottom), median (middle) and third quartile (top). Asterisks indicate significance values for means comparison, * p<0.01, ** p<0.001, *** p<0.0001, (Supplementary Material, Tables 6.1-3 and Tables 7.1-3).
Table 1. Quantification of behavioral phenotypes and mortality for *syngap1* and *shank3* morphants.

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Table 2. Statistical results for *SYNGAP1* cell death ANOVA.
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<th>Neuronal Region</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig. (p&lt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
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<td></td>
<td></td>
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<tr>
<td>Between Subjects</td>
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<td>151</td>
<td>65.8</td>
<td>11.5</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
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See Supplementary Material, Tables S9.1-3 for means comparison analysis
Table 3. Statistical results for *SHANK3* cell death ANOVA.

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<th>Neuronal Region</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>Sig. (p&lt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Midbrain</td>
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</table>

See Supplementary Material, Tables S10.1-3 for means comparison analysis.
Table 4. Measurements of anti-GABA and vglut:DsRed for morphant midbrain sections.

<table>
<thead>
<tr>
<th></th>
<th>GABA (# cells)</th>
<th>vGluT (% μm²)</th>
<th>Cortical Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoMO</td>
<td>22.2 ± 5.42</td>
<td>14.5 ± 3.51</td>
<td>5.41x10⁴ ± 4.81x10³</td>
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<tr>
<td>syngap1b MO</td>
<td>8.6 ± 5.56 *</td>
<td>12.7 ± 3.22</td>
<td>2.71x10⁴ ± 9.42x10³ ***</td>
</tr>
<tr>
<td>shank3a MO</td>
<td>10.0 ± 6.36 *</td>
<td>13.32 ± 2.02</td>
<td>3.45x10⁴ ± 3.19x10³ ****</td>
</tr>
</tbody>
</table>

Students t-test significance; * p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001. Sample sizes; CoMO n=6, for syngap1b MO n=6 and shank3a MO n=6.
Table 5. Measurements of anti-GABA and vglut:DsRed for morphant hindbrain sections.

<table>
<thead>
<tr>
<th></th>
<th>GABA (# cells)</th>
<th>vGluT (% μm²)</th>
<th>Cortical Area (μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CoMO</td>
<td>20.7 ± 5.0</td>
<td>16.6 ± 5.16</td>
<td>2.15x10^4 ± 1.22x10^3</td>
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<tr>
<td>syngap1b MO</td>
<td>15 ± 4.0</td>
<td>8.26 ± 3.4 *</td>
<td>1.63x10^4 ± 2.36x10^3 **</td>
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<tr>
<td>shank3a MO</td>
<td>14.2 ± 2.8 *</td>
<td>8.44 ± 4.05 *</td>
<td>1.14x10^4 ± 1.68x10^3 ****</td>
</tr>
</tbody>
</table>

Students t-test significance; * p<0.05, ** p<0.01, *** P<0.001, **** p<0.0001. Sample sizes; CoMO n=6, for syngap1b n=5 and shank3a n=6.