Hypokinesia and Reduced Dopamine Levels in Zebrafish Lacking β- and γ1-Synucleins*  

Background: Synucleins are implicated in Parkinson disease pathogenesis, but their functions are incompletely understood. α-Synuclein is strongly implicated in the pathogenesis of Parkinson disease. However, the normal functions of synucleins and how these relate to disease pathogenesis are uncertain. We characterized endogenous zebrafish synucleins in order to develop tractable models to elucidate the physiological roles of synucleins in neurons in vivo. Three zebrafish genes, sncb, sncg1, and sncg2 (encoding β-, γ1-, and γ2-synucleins respectively), show extensive phylogenetic conservation with respect to their human paralogues. A zebrafish α-synuclein orthologue was not found. Abundant 1.45-kb sncb and 2.7-kb sncg1 mRNAs were detected in the CNS from early development through adulthood and showed overlapping but distinct expression patterns. Both transcripts were detected in catecholaminergic neurons throughout the CNS. Zebrafish lacking β-, γ1-, or both synucleins during early development showed normal CNS and body morphology but exhibited decreased spontaneous motor activity that resolved as gene expression recovered. Zebrafish morphology but exhibited decreased spontaneous motor behavior. Zebrafish models will allow further elucidation of the molecular physiology and pathophysiology of synucleins in vivo.

Results: Zebrafish lacking β- and γ1-synucleins showed hypokinesia and decreased dopamine levels. These abnormalities were rescued by human α-synuclein.

Conclusion: Synucleins are necessary for spontaneous movement and dopaminergic function.

Significance: A key functional role for synucleins in dopamine neurons may be relevant to Parkinson disease pathogenesis.

Synucleins are small proteins of 100–140 amino acids that are expressed abundantly in neuronal presynaptic terminals (reviewed in Ref. 1). The three members of the synuclein family, α-, β-, and γ-synuclein, are encoded by separate genes and are widely conserved throughout the vertebrate subphylum. Pathogenic missense (2–4) and gene dose (5, 6) mutations in the SNCA gene encoding α-synuclein are an uncommon cause of familial parkinsonism. Convergent lines of evidence suggest that α-synuclein is also centrally involved in the pathogenesis of the common sporadic form of Parkinson disease (PD). The pathological hallmark intraneuronal inclusions of sporadic PD, Lewy bodies, contain insoluble fibrillar aggregates of α-synuclein (7). Furthermore, genome-wide association studies (8–10) show an association between genetic variants at the SNCA locus and risk of developing PD. Recent studies support the possibility that SNCA variants influencing PD risk may alter α-synuclein expression (11), suggesting that alterations in α-synuclein levels may predispose susceptible neuronal groups to develop pathology in PD. It is currently unclear whether pathogenic involvement of α-synuclein in PD reflects quantitative changes in the activity of its normal cellular functions or the emergence of new pathological functions as its cellular abundance changes. Understanding the normal functions of synucleins is thus an important goal, because this might elucidate aspects of pathogenesis and facilitate identification of therapeutic targets in PD.

Prominent localization of synucleins at presynaptic terminals suggests that they play an important role in synaptic transmission. Mice lacking α-synuclein (12–16), β-synuclein (17), or γ-synuclein (18) show normal development, survival, CNS morphology, synaptic protein expression, synaptic density, and behavior. Changes in the number of dopaminergic neurons (19), striatal dopamine levels and dopamine release (14), and synaptic vesicle pools (16) have been reported in individual

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1 A R.I.MED scholar.

2 To whom correspondence should be addressed: Dept. of Neurology, University of Pittsburgh, 7015 BST-3, 3501 Fifth Ave., Pittsburgh PA 15213. Tel: 412-648-8480; E-mail: eab25@pitt.edu.
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lines of SNCA−/− animals and may reflect variations in genetic background. Synucleins show overlapping expression patterns, raising the possibility that minor abnormalities in single knock-out animals are attributable to functional compensation by other synucleins. Mild dopaminergic abnormalities and up-regulation of β-synuclein were found in animals lacking both α- and γ-synucleins (19). Loss of α- and β-synucleins together caused changes in the expression of presynaptic proteins, including up-regulation of γ-synuclein, and a modest reduction in striatal dopamine levels (17). Loss of all three synucleins gave rise to progressive motor impairment; reduced survival; changes in synaptic morphology, physiology, and protein expression; and reduced SNARE complex formation (20, 21) in addition to reduced striatal dopamine content and altered dopamine release (22). These studies show that synucleins are essential for long term maintenance of synapses in the vertebrate CNS, although the molecular basis of these observations and their relevance to Parkinson disease remain undefined. An accessible model allowing mechanistic studies in the intact vertebrate nervous system in vivo might be valuable, allowing further definition of the role of synucleins in the molecular physiology of presynaptic terminals.

The zebrafish is a powerful model for studying the vertebrate nervous system. Larvae can be manipulated to be optically transparent, enabling direct visualization of neurons (23, 24), allowing further definition of the role of synucleins in the intact vertebrate nervous system. Larvae can be subjected to unbiased phenotype-based genetic (31, 32) and chemical (33, 34) screening approaches in order to discover novel molecular insights. The purpose of the present study was to identify and characterize endogenous zebrafish synucleins to allow studies of their functions in vivo and to facilitate the development of zebrafish models of Parkinson disease. Here we report that zebrafish do not express α-synuclein. Zebrafish β- and γ1-synucleins are expressed widely in CNS neurons, including dopamine neurons, and are necessary for the development of normal motor and dopaminergic function in zebrafish larvae.

EXPERIMENTAL PROCEDURES

Zebrafish—Experiments were carried out in accordance with National Institutes of Health guidelines and Institutional Animal Care and Use Committee approval. Adult strain AB zebrafish were maintained at 28.5 °C and euthanized by deep tricaine anesthesia followed by exposure to ice-cold water. Embryos were raised in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄), supplemented where necessary with 0.003% 1-phenyl-2-thiourea to inhibit pigmentation.

Synteny Analysis—Online tools (NCBI genome viewer and Cinteny (35), both available on the World Wide Web) were used to evaluate syntenic relationships. Proposed orthologues were verified by multiple sequence alignments, and synteny maps were drawn manually.

RT-PCR and 5’-RACE—First strand cDNA was generated by oligo(dT)-primed reverse transcription of total RNA from whole adult zebrafish brains or pooled embryo lysate (SuperScript III, Invitrogen). Nested 5’-RACE was carried out (RLM-RACE, Ambion, Austin, TX) as described previously (24, 36, 37). Primer sequences are listed in supplemental Table 1.

RNA Hybridization and Immunofluorescence—Northern blots (36), whole mount RNA in situ hybridization (24), mRNA in situ hybridization to brain cryosections (24), double label ISH/IHC (38), and indirect immunofluorescence and confocal microscopy (24, 39) were carried out as reported in our previous work. Rabbit anti-α-synuclein (1:300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used to detect human α-synuclein in tissue sections.

Morpholino Oligonucleotides—Antisense morpholino oligonucleotides (MOS; Gene Tools LLC, Philomath, OR) were designed to inhibit zebrafish synuclein gene expression by targeting splice signals in the primary transcripts. Expression of sncb was disrupted by MOS targeted against splice donor sites of exons 2 and 3 (sncb E2/I2, aatgttgcatgacttacCTGTTGCA; sncb E3/I3, ttgccagatctctatCGTITGCA; sequence complementary to the 5’-end of each exon is shown in capital letters). Expression of sncg1 was disrupted by MOS targeted against the splice donor sites of exons 1 and 3 (sncg1 E1/I1, tatacatacgatccACACACAC; sncg1 E3/I3, ctggattcctactcatCGGGGT). Control MOSs (random MO (N25) and non-targeting MO CCTTTCACCTAGTTCAATTTATA) were used to exclude nonspecific effects arising from morpholino exposure.

3 ml of MO dissolved in 1× Danieau Buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, 5.0 mM HEPES, pH 7.6) was injected into the yolk sac at the 1–2-cell stage. For sncb, 3 ng of sncb E3/I3 MO and 5 ng of sncb E2/I2 MO were injected (β MO). For sncg1, 4 ng of each of sncg1 E1/I1 and sncg1 E3/I3 were injected (γ1 MO). To target both sncb and sncg1, all four MOs were injected (β + γ1 MO), total dose 14 ng. Control MO was injected at 14 ng/embryo.

mRNA Rescue—A NotI/XhoI fragment of pRES2-EGFP (Clontech, Mountain View, CA) was inserted into the Xbal/Xhol sites of pCS2+ (40) to generate plasmid pCS-IG. Human α-synuclein cDNA was amplified from NT2 cells by RT-PCR, sequenced, and inserted into the Xhol/Xmal sites of pCS-IG to generate pCS-syn-IG. pCS-IG and pCS-syn-IG were linearized at the NotI site 3’ to the poly(A) signal, and mRNA was transcribed from the Sp6 promoter (mMessage Machine, Ambion, Austin, TX). Intracellular injection of 1–1.25 nl of mRNA solution (250 pg/nl mRNA, 0.25% phenol red, 120 mM KCl, 20 mM HEPES, pH 7.4) was carried out at the single cell stage, followed by MO injection into the yolk sac as above.

Western Blot—Western blots were carried out as described previously (36, 39). Proteins were transferred to PVDF membrane (Immobilon, Millipore, Billerica, MA), and blots were blocked in Odyssey Blocking Buffer (LI-COR, Lincoln, NE). Primary antibodies were as follows: α-synuclein (mouse; 1:1000; BD Biosciences) and β-actin (rabbit; 1:2000; Sigma). Secondary antibodies conjugated to different infrared fluorophores (anti-mouse IRdye®800 and anti-rabbit IRdye®680; 1:10,000 each; LI-COR) allowed simultaneous detection of both proteins using a two-channel infrared imaging system (LI-COR).

Motor Analysis—Larval movement was analyzed using a proprietary system (Zebralab; ViewPoint Life Sciences, Montreal,

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Canada) as reported previously (34). Zebralab software was used in “quantization mode” with detection sensitivity set to 16 and with burst and freezing activity thresholds set to 10 and 2, respectively. Groups of 15 embryos were analyzed in 12-well plates containing 3 ml of E3 buffer/well. Spontaneous activity was measured for an 8-h period daily, from 24 h postfertilization (hpf) to 7 days postfertilization (dpf).

Neurochemical Measurements—Neurochemical measurements were performed by adapting a previously reported method (41). 30 larvae were sonicated in 150 μl of 4 M perchloric acid, 0.1% Na2S2O7, 0.1% EDTA at 4 °C. The sample was centrifuged at 17,000 × g at 4 °C, and the supernatant was filtered through a 0.22-μm nylon membrane (Spin-X, Corning Glass). 25 μl was loaded into a Waters 2695 HPLC separation module (Waters, Milford, MA). The HPLC mobile phase consisted of 0.06 M sodium phosphate monobasic, 0.03 M citric acid, 8% methanol, 1.1 mM 1-octanesulfonic acid, 0.1 mM EDTA, 0.22 μM perchloric acid, pH 3.5. The flow rate was 1.0 ml/min. Neurotransmitters were separated on a Waters XBridge C18 4.6 × 150-mm column, particle size 3.5 μm, at 34 °C, and detected using a Waters 2465 electrochemical detector with a glassy carbon electrode set at 750 mV, referenced to an ISAAC electrode. Neurotransmitters were quantified as pg/larva, using a standard curve generated from injection of high purity standards. Each data point was then normalized to the value for wild type animals in the same experiment.

Statistical Analysis—Quantitative data were shown to be normally distributed by Pearson’s and D’Agostino’s tests and were analyzed using parametric statistical tests. All experiments were repeated at least in triplicate. Graphs show means ± S.E. One-way ANOVA was carried out after confirming the assumption of equal variance using Bartlett’s test and was followed by Dunnett’s post hoc test to evaluate statistically significant differences between test groups.

RESULTS

Zebrafish Synucleins—Identification of zebrafish synucleins was recently reported by two other groups (42, 43). We independently cloned the same genes at the start of this study (not shown). sncb encodes the zebrafish orthologue of human β-synuclein, and sncg1 and sncg2 encode dual paralogues of human γ-synuclein. Zebrafish synucleins show extensive sequence similarity to human synucleins, except that the C termini of the two zebrafish γ-synucleins are relatively divergent (Fig. 1). We did not identify an orthologue of human α-synuclein in zebrafish genomic, mRNA, or expressed sequence tag databases, which was unexpected because α-synuclein orthologues have been identified in mammals, birds, reptiles, amphibians, and other fish species. Despite employing a variety of RT-PCR and RACE strategies to amplify a putative sncg transcript (supplemental Table 4), we did not find evidence of a zebrafish α-synuclein orthologue expressed in the CNS.

In order to further understand how human and zebrafish synuclein genes are related, we constructed synteny maps (Fig. 2). Two of the genes flanking the sncb gene on zebrafish chromosome 13 are orthologues of genes flanking the human SNCB gene on chromosome 5 (Fig. 2A), suggesting that the genes are derived from a common ancestral locus. A large genomic region surrounding zebrafish sncg1 and sncg2 is duplicated and is syntenic with the human genomic region containing SNCG (Fig. 2B). These observations suggest that sncg1 and sncg2 are derived from duplication of an ancestral locus shared with human SNCG. Zebrafish orthologues of genes immediately flanking human SNCA were not found in the available zebrafish genome sequence, whereas the wider genomic region in the human showed two blocks of genes that were syntenic to different regions of the zebrafish genome (Fig. 2C). It is possible that the region between these syntenic blocks was deleted from an ancestral chromosome during evolution, accounting for the lack of zebrafish genomic or mRNA sequences with homology to human SNCA.

mRNA Transcripts and Genomic Organization of Zebrafish Synucleins—Zebrafish sncb and sncg1 transcripts were detected abundantly in adult brain by Northern blot (Fig. 3A). Each gene gave rise to a single mRNA species of 1.45 kb (sncb) or 2.7 kb (sncg1). sncg2 was alternatively spliced to yield two transcripts encoding isoforms with divergent C termini; although both transcript variants were amplified from CNS by PCR (Fig. 3B), we were unable to identify sncg2 transcripts in the CNS by Northern blot hybridization or RNA in situ hybridization and conclude that they are present at low abundance. The remainder of this study focused on sncb and sncg1 encoding the predominant CNS synucleins in zebrafish.

5′-RACE (Fig. 3C), using the tobacco acid pyrophosphatase (TAP) method (as described previously (24, 36, 37)), was employed to map the 5′-ends of the sncb and sncg1 transcripts. TAP+ and RT-PCR controls verified that the boundary between the RACE adapter and the cDNA sequence in the RACE products occurred at the mRNA cap site. Both sncb and sncg1 promoter regions lack canonical TATA motifs and show multiple transcriptional start sites from motifs with homology to the initiator consensus 5′-YYA+NWYY-3′ (44) (Fig. 3, D and E). We next examined the genomic organization of sncb, sncg1, and sncg2 by comparing cDNA sequences with the zebrafish genomic sequence (Fig. 3, F–H, and supplemental Tables 2 and 3). Like its human orthologue, sncb contains six exons, the first of which is non-coding. Similar to their human
paralogue, both sneg1 and sneg2 contain five exons. The positions and phases of the splice boundaries in sneb, sneg1, and sneg2 are conserved with respect to their human paralogues, providing further evidence of a common origin (supplemental material).

Expression Patterns of Zebrafish Synucleins—The expression patterns of zebrafish synucleins were determined by RNA in situ hybridization. As reported previously during early development (42, 45), sneb and sneg1 were expressed in the CNS, whereas sneg2 was found in the notochord (Fig. 4, A–C). In the adult zebrafish CNS, both sneb and sneg1 were expressed in cells with neuronal morphology, widely distributed throughout gray matter of the brain and spinal cord. However, their topographical expression patterns differed significantly; sneb was more robustly expressed rostrally, whereas sneg1 expression was more prominent caudally (Fig. 4, D and E). Differences in the expression patterns of sneb and sneg1 in specific brain regions are illustrated in Fig. 4F. Whereas prominent sneb expression was detected in the olfactory bulb and dorsal telencephalon, sneg1 was not expressed at high levels in these regions. Conversely, robust sneg1 expression in the medulla oblongata and habenula was accompanied by weak expression of sneb in these areas. Even within some brain regions where there was overlap between sneb and sneg1 expression, the two genes were expressed in different cellular populations. For example, within the cerebellum, sneb was expressed within granule cells, whereas sneg1 was expressed most prominently in the Purkinje cell layer (Fig. 4G).

We next examined expression of sneb and sneg1 in catecholaminergic neurons in the zebrafish CNS. In the absence of available antibodies to zebrafish β- or γ1-synucleins, we used a double label ISH/IHC technique, allowing us to detect sneb or sneg1 and tyrosine hydroxylase simultaneously in the same tissue sections. Examples are shown in Fig. 4H; dopaminergic neurons of the periventricular hypothalamus were identified by their morphology and by robust tyrosine hydroxylase immunoreactivity in their processes. Strong expression of sneb (Fig. 4H, left) and sneg1 (right) was also evident in the perinuclear cytoplasm. Using this method, we examined each of the major catecholaminergic cell groups within the olfactory bulb, telencephalon, diencephalon, and hindbrain. With the exception that sneg1 expression was relatively weak in the olfactory bulb, all tyrosine hydroxylase-immunoreactive groups also expressed both sneb and sneg1. These data suggest that sneb and sneg1 may be co-expressed in catecholaminergic neurons.

β- and γ1-Synucleins Are Not Required for Early Embryogenesis or CNS Morphogenesis—We next asked whether perturbing expression of sneb or sneg1 using antisense MOs would result in a neurodevelopmental phenotype that might be
FIGURE 3. Zebrafish synucleins: transcripts, promoters, and genomic organization. A, Northern blots containing total RNA from adult zebrafish brain were hybridized with cRNA probes complementary to sncb (left) or sncg1 (right). The positions of molecular size markers (kb) are shown to the left of the blots, and the positions of the 28 and 18 S ribosomal RNA bands are shown to the right.

B, total RNA derived from adult brain or embryos at 48 hpf was subjected to RT-PCR using primers complementary to exons 3 and 5 of sncg2. The picture shows an ethidium-stained agarose gel under UV illumination following electrophoretic separation of the products. Bands corresponding to sncg2_tv1 (lacking exon 4) and sncg2_tv2 (containing exon 4) are labeled to the right of the picture.

C, 5’RACE was used to map the transcriptional start sites of sncb (lanes 1, 3, 5, and 7) and sncg1 (lanes 2, 4, 6, and 8). The picture shows an ethidium-stained agarose gel under UV illumination following electrophoretic separation of PCR products. 5’RACE (lanes 1–4) and RT-PCR (lanes 5–8) were carried out using cDNA template derived from samples in which RNA was either treated with tobacco acid pyrophosphatase (TAP+/H11001; lanes 1, 2, 5, and 6) or received no treatment (TAP-/H11002; lanes 3, 4, 7, and 8) prior to adapter ligation.

D and E, the promoter regions of sncb (D) and sncg1 (E) are shown. The arrows indicate transcriptional start sites identified by 5’RACE; initiator sequences are highlighted in gray, and the major start sites of each transcript are highlighted in black. The splice donor consensus of sncb exon 1 is underlined. A pyrimidine-rich sequence in the sncg1 5’-flanking region is underlined. F–H, the genomic organization of sncb (F), sncg1 (G), and sncg2 (H) are shown schematically. The lower part of each diagram depicts the genomic sequence showing the position and number of each exon. The upper part shows the major transcripts arising from each gene and the locations of the open reading frames (shaded in black and labeled ATG→TAG). *, splice donor sequences in sncb and sncg1 that were targeted by antisense morpholino oligonucleotides.
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informative regarding the normal functions of β- and γ1-synucleins. Antibodies to zebrfish synucleins are not available, so verification of translation-inhibiting MOs was not possible; consequently, we inhibited gene expression by targeting pre-mRNA splice sites because splicing could be verified by RT-PCR. Pairs of MOs targeting two splice boundaries in each transcript greatly reduced expression, such that sncb or sncg1 mRNA could not be detected by RT-PCR up to 72 hpf (Fig. 5, A and B). We refer to the pair of MOs targeting sncb as β MO and the pair of MOs targeting sncg1 as γ1 MO. As a control for nonspecific effects of MOs, we used both non-targeting and random MOs (termed Ctrl MO in subsequent figures) at the
same concentrations. These did not affect expression of either sncb or sncg1 (Fig. 5, A and B).

Zebrafish lacking β-, γ1-, or both synucleins were viable and showed normal development of the basic body plan, internal organs, and circulation (Fig. 5C). In order to examine the morphology of the nervous system in more detail, we employed Tg(eno2:egfp) zebrafish, which express GFP in a pan-neuronal pattern (24), allowing delineation of CNS structure and exam-

![FIGURE 5. Morpholino oligonucleotides targeting expression of sncb and sncg1 during development. A, morpholino oligonucleotide targeting of sncb. RNA was harvested from zebrafish embryos at 24 hpf (lanes 1–3), 48 hpf (lanes 4–6) and 72 hpf (lanes 7–9) and subjected to RT-PCR analysis using primers to sncb (top) or bactin1 (bottom). The images show UV-illuminated ethidium-stained agarose gels following electrophoretic separation of the PCR products. Uninjected embryos (WT; lanes 1, 4, and 7) were compared with embryos injected at the single cell stage with MOs targeting the splice donor sequences of exons 2 and 3 of the primary sncb transcript (β MO; lanes 2, 5, and 8) or a non-targeting control MO (Ctrl MO; lanes 3, 6, and 9). The positions of molecular size markers (bp) are shown to the left of the image. The positions of bands corresponding to sncb mRNA and an sncb transcript lacking exon 3 are labeled to the right of the image. B, morpholino oligonucleotide targeting of sncg1. RNA was harvested from zebrafish embryos at 24 hpf (lanes 1–3), 48 hpf (lanes 4–6), and 72 hpf (lanes 7–9) and subjected to RT-PCR analysis using primers to sncg1 (top) or bactin1 (bottom). Uninjected embryos (WT; lanes 1, 4, and 7) were compared with embryos injected at the single cell stage with MOs targeting the splice donor sequences of exons 1 and 3 of the primary sncg1 transcript (γ1 MO; lanes 2, 5, and 8) or a non-targeting control MO (Ctrl MO; lanes 3, 6, and 9). C, morphology of zebrafish lacking β- or γ1-synuclein. Zebrafish embryos were injected at the single cell stage with injection buffer only (buffer), a non-targeting control MO (Ctrl MO), or MOs targeting sncb (β MO), sncg1 (γ1 MO), or both sncb and sncg1 (β + γ1 MO). Bright field lateral images of larvae at 72 hpf and dorsal views of the head region are shown alongside epifluorescence images from Tg(eno2:egfp) (24) larvae at the same time points in order to show the structure of the CNS.]

![FIGURE 4. Expression of zebrafish synucleins during development and in the adult CNS. A–C, whole mount RNA in situ hybridization was carried out in zebrafish embryos at 24 hpf using cRNA probes to sncb (A), sncg1 (B), or sncg2 (C). Hybridized probe was revealed using a chromogenic substrate with a purple/blue reaction product. Structures expressing each transcript are labeled. FB, forebrain; HB, hindbrain; SC, spinal cord; NC, notochord. D and E, RNA in situ hybridization was carried out on parasagittal sections from fixed adult brains using cRNA probes specific to sncb (D) and sncg1 (E). Hybridized probe was revealed using a chromogenic substrate with a purple/blue reaction product. The images show low magnification micrographs to illustrate the topographical expression patterns of the transcripts. The scale for both images is shown to the right of D. Structures expressing each transcript are labeled in each image. OB, olfactory bulb; D, dorsal telencephalon; TeO, optic tectum; Ha, habenula; PC, cerebellar Purkinje cell layer; IRF, inferior reticular formation. F and G, adult brain sections processed identically to those shown in D and E are shown at higher magnification in order to illustrate regions in which sncb (top row) and sncg1 (bottom row) showed different expression patterns. The anatomical region is labeled above each pair of images, and the scale for both micrographs is shown in the upper of the two. In G, the cerebellum is shown at two different magnifications. M, molecular layer; P, Purkinje cell layer; G, granule cell layer. H, two-color RNA in situ hybridization/immunohistochemistry (38) was used to examine expression of synuclein mRNAs in dopaminergic neurons. In each image, RNA in situ hybridization was carried out using cRNA probes complementary to sncb (left) or sncg1 (right), and hybridized probe was detected using a chromogenic reaction with a blue/purple product. The sections were then labeled using an antibody to tyrosine hydroxylase (64), and bound antibody was detected using a second chromogenic reaction with a red product. Examples are shown of dopaminergic neurons in the periventricular hypothalamus that showed expression of sncb or sncg1 mRNA in their cell bodies (purple arrows) and tyrosine hydroxylase (TH) in their processes (red arrows). The scale for both images is shown in the lower right corner of the left panel.]
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In order to firmly establish whether these motor abnormalities were attributable to loss of synucleins, we next asked whether this hypokinetic phenotype could be rescued by an exogenous synuclein. Human α-synuclein was expressed in zebrafish embryos by microinjecting a bicistronic mRNA encoding both α-synuclein and GFP. mRNA encoding GFP alone was used as a control. Because the IRES element functions weakly in zebrafish (not shown), human α-synuclein expression was verified by other methods. Western blot showed a single 16-kDa α-synuclein immunoreactive band specific to microinjected embryos; immunofluorescence demonstrated robust α-synuclein expression throughout microinjected embryos, including the neural tube and developing retina (Fig. 6C). Expression of either GFP or human α-synuclein did not alter WT larval motor activity (Fig. 6D). Reduced motor activity at 4 dpf was seen in GFP-expressing larvae lacking β-, γ1-, or β- and γ1-synucleins, similar to results in larvae expressing no exogenous mRNA. In contrast, animals expressing human α-synuclein showed normal levels of motor activity in the absence of β- or γ1-synuclein. In the absence of both β- and γ1-synucleins, it was necessary to express a higher level of human α-synuclein in order to prevent loss of spontaneous movement. These data show that the hypokinetic abnormality observed after loss of β-, γ1-, or β- and γ1-synucleins is attributable to loss of synucleins rather than an artifact of the experimental system. Together, these findings demonstrate that β- and γ1-synuclein expression in neurons is essential for physiological functions that underlie normal motor behavior during development.

β- and γ1-Synucleins Are Required for Development of the Dopamine System—We next addressed the hypothesis that the motor phenotype resulting from loss of β- or γ1-synuclein was attributable to abnormalities of the dopamine system. We first asked whether development of dopaminergic neurons differed between controls and zebrafish lacking β-, γ1-, or both synucleins. RNA in situ hybridization was carried out at 48, 72, and 96 hpf, using a cRNA probe to slc6a3 (dopamine transporter, dat), which is specifically expressed in dopamine neurons (38, 46) (Fig. 7A). No qualitative differences were observed in the distribution or staining intensity of slc6a3-expressing cells between controls and larvae lacking β-, γ1-, or both synucleins. However, quantification by an observer blinded to MO exposure showed a modest reduction in the number of slc6a3+ cells in larvae lacking both β- and γ1-synucleins at 48 hpf (Fig. 7B). This abnormality had recovered by 72 hpf, such that there was no difference in the number of slc6a3+ cells between WT larvae and those exposed to β + γ1 MO (Fig. 7C). Expression of GFP or human α-synuclein did not alter the number of slc6a3+ neurons in control larvae (Fig. 7D). However, expression of human α-synuclein was sufficient to prevent a reduction in the number of slc6a3+ cells in the absence of β- and γ1-synucleins, whereas GFP expression did not rescue this phenotype. These data show that both β- and γ1-synuclein are necessary for early development or differentiation of dopamine neurons. However, there was a normal complement of dopamine neurons by 4 dpf, when the hypokinetic phenotype in zebrafish lacking synucleins was most prominent.

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β- and γ1-Synucleins Are Required for Development of the Dopamine System—We next addressed the hypothesis that the motor phenotype resulting from loss of β- or γ1-synuclein was attributable to abnormalities of the dopamine system. We first asked whether development of dopaminergic neurons differed between controls and zebrafish lacking β-, γ1-, or both synucleins. RNA in situ hybridization was carried out at 48, 72, and 96 hpf, using a cRNA probe to slc6a3 (dopamine transporter, dat), which is specifically expressed in dopamine neurons (38, 46) (Fig. 7A). No qualitative differences were observed in the distribution or staining intensity of slc6a3-expressing cells between controls and larvae lacking β-, γ1-, or both synucleins. However, quantification by an observer blinded to MO exposure showed a modest reduction in the number of slc6a3+ cells in larvae lacking both β- and γ1-synucleins at 48 hpf (Fig. 7B). This abnormality had recovered by 72 hpf, such that there was no difference in the number of slc6a3+ cells between WT larvae and those exposed to β + γ1 MO (Fig. 7C). Expression of GFP or human α-synuclein did not alter the number of slc6a3+ neurons in control larvae (Fig. 7D). However, expression of human α-synuclein was sufficient to prevent a reduction in the number of slc6a3+ cells in the absence of β- and γ1-synucleins, whereas GFP expression did not rescue this phenotype. These data show that both β- and γ1-synuclein are necessary for early development or differentiation of dopamine neurons. However, there was a normal complement of dopamine neurons by 4 dpf, when the hypokinetic phenotype in zebrafish lacking synucleins was most prominent.

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Finally, we asked whether abnormalities of monoaminergic neurochemistry might explain the hypokinesia resulting from loss of β- or γ1-synucleins. We examined steady-state whole larval dopamine levels at 7 dpf, which was the earliest time point at which we could measure neurotransmitters with sufficiently low variability to enable detection of differences between multiple experimental groups. Although gene expression had recovered by this time point, zebrafish lacking both β- and γ1-synucleins earlier in development showed significantly reduced dopamine levels at 7 dpf compared with Ctrl MO-injected animals (Fig. 7E). None of the other experimental groups showed significant differences from controls. Transient expression of GFP or human α-synuclein early in development did not change steady state levels of dopamine in WT larvae at 7 dpf. However, α-synuclein but not GFP prevented the loss of dopamine that occurred in the absence of zebrafish β- and γ1-synucleins.
nucleins (Fig. 7F). These data show that early developmental expression of both β- and γ1-synucleins is necessary for establishment of normal dopamine levels. Interestingly, 5-hydroxytryptamine levels were not reduced in larvae lacking synucleins (Fig. 7G), suggesting that the relevant function of synucleins in this context may be specific to dopaminergic neurons.

DISCUSSION

A genomic duplication occurred early in the evolution of ray-finned fish (47) and probably accounts for the presence of two snca genes in zebrafish, similar to fugu, medaka, salmon, and stickleback (48). In some cases, duplicated genes have complementary roles, sharing multiple functions of the ancestral gene (49). The dual zebrafish paralogues of γ-synuclein show distinct temporal and spatial expression patterns; this may have allowed segregation of neural and non-neural functions of γ-synuclein into separate proteins and provided evolutionary pressure to retain both genes. Conversely, our data suggest that an ancestral snca gene and its flanking genes were lost during zebrafish evolution. The putative deletion appears to have occurred more recently than the duplication of snca, because a number of other fish species, including salmon and fugu, harbor duplicate snca genes in addition to snca and sncb. A precedent for spontaneous loss of snca has been described in a subpopulation of the C57BL/6 strain of mice that developed a de novo deletion of the SNCA locus (15). It is likely that other synuclein family members provide compensatory functions accommodating the loss of a single synuclein gene; mice lacking α-, β-, and γ-synucleins (20–22) showed more severe phenotypes than animals lacking one or two synucleins (17, 18), and abnormalities in α-, β-, and γ-synuclein null mice were mitigated by expression of human α-synuclein (21). Here we provide further evidence of cross-functionality between dif-
different synucleins by showing that loss of zebrafish β- and γ1-synucleins gave rise to a more severe phenotype than loss of one or other synuclein and that abnormalities arising from loss of either protein were rescued by expression of human α-synuclein.

By using an assay sensitive to motor function early in development, we demonstrated that transient abrogation of β- or γ1-synuclein expression suppressed normal spontaneous motor activity in larval zebrafish. There was no accompanying alteration in the structure of the body or CNS, and we conclude that the mechanisms underlying the observed hypokinesia are likely to involve a disturbance of neuronal function. Resolution of hypokinesia was temporally correlated with the gradual recovery of gene expression after 72–96 hpf, suggesting that synucleins dynamically regulate aspects of neuronal function governing initiation of spontaneous movement. Abnormalities of synaptic transmission have been demonstrated in murine synuclein knock-out models (20, 21), and it is possible that similar defects arise in circuits mediating initiation of spontaneous movement in zebrafish lacking β- or γ1-synucleins. Given the importance of dopaminergic mechanisms in human hypokinetic movement disorders associated with synucleinopathies and the possibility that synucleins have important specific functions in dopamine neurons (22), we evaluated the role of β- and γ1-synucleins in the dopaminergic system. Both proteins were expressed in dopamine neurons throughout the CNS, and loss of both proteins delayed the appearance of a full complement of slc6a3γ1-expressing neurons in the developing brain. Although the number of slc6a3γ1-neurons was normal at 4 dpf, it is possible that delayed differentiation of dopaminergic neurons might contribute to later abnormalities of motility, by affecting the formation of motor circuits. Even in the presence of a normal number of dopamine neurons, we found evidence of disturbed dopaminergic function; at 7 dpf, zebrafish exposed to β + γ1 MOs showed decreased dopamine levels, similar to previous studies in synuclein gene knock-out mice (17, 22). Because reproducible measurements of dopamine levels were not technically feasible at earlier time points, we did not exclude the possibility that reduced dopamine levels were also present at the time points where the most prominent hypokinesia was observed. The development of zebrafish lines harboring stable null alleles of sncb and sncg1 will allow clarification of the relationship between synucleins, dopamine levels, and motor phenotypes later in development when neurochemical measurements are more robust. Stable sncb−/− and sncg1−/− lines will also allow unbiased screening approaches to isolate modifiers of the hypokinetic synuclein-null phenotype that might be informative concerning genetic interactions of synucleins in vertebrate neurons in vivo. Previous screens for genetic modifiers have been carried out in yeast expressing human α-synuclein (50–52), and the role of a subset of identified modifiers was confirmed in fly (53) and worm (52) models. Although these studies provide information about the pathways involved in cellular responses to ectopic α-synuclein overexpression, the physiological roles of synucleins may depend on specific protein interactions absent from organisms lacking synucleins natively. Consequently, zebrafish models may contribute important new insights into synuclein function at the vertebrate presynaptic terminal.

There has been increasing interest in the development of zebrafish models of human neurodegenerative disease for therapeutic target identification and drug discovery in vivo (28). Transient knockdown of a number of different zebrafish orthologues of human genes implicated in parkinsonism resulted in dopaminergic phenotypes, including loss of neurons (54–56), altered function (56), or susceptibility to toxins (55, 57). Furthermore, zebrafish exposed to toxins implicated in PD pathogenesis (41, 58–61) showed loss of dopamine neurons, dopamine content, or dopaminergic function. Together, these data have provided encouragement that it might be possible to recapitulate aspects of PD pathogenesis accurately in zebrafish, an essential prerequisite for the development of useful models. We report here that zebrafish lack endogenous α-synuclein, which may cast doubt on whether key aspects of PD pathogenesis can be reproduced in a zebrafish model. α-Synuclein pathology is not prominent in some types of PD (62, 63), and it is currently unclear whether α-synuclein is necessary for PD pathogenesis in all cases. However, we consider the construction of transgenic zebrafish lines expressing physiological levels of human or fish α-synucleins an important priority. Because the zebrafish natively presents an sncα-null background, comparison of α-synuclein-expressing transgenic lines with controls should clarify the role of α-synuclein in neuronal loss caused by PD-relevant environmental and genetic triggers.

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