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Generation + characterization of alpha-synuclein transgenic zebrafish

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Generation + Characterization of α-synuclein transgenic zebrafish

by

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Generation and characterization of α-synuclein transgenic zebrafish

Summary:

Parkinson's disease, the second most common neurodegenerative disease, is characterized by loss of DA neurons and presence of α-synuclein (α-syn) aggregates in surviving neurons. The α-syn protein plays a central role in disease pathology, but attempts to define α-syn's role has proven to be difficult. Interestingly, simple over-expression of α-syn in *Drosophila* recreates many of the symptoms of PD (Feany & Bender 2000). This fruit fly model of PD has proven invaluable in defining α-syn's role in PD pathology, however the fruit fly's low homology with humans remains a limiting factor in the utility of this model. In this study I am generating a zebrafish model of PD by over-expressing α-syn. Such a model can elucidate the mechanisms of PD pathology and α-syn toxicity, as well as be used for finding new therapies for PD.

Rationale:

Parkinson's disease (PD) is the 2nd most common neurodegenerative disorder, afflicting 1-2% of the population over the age of 65. Symptoms of PD include muscle rigidity, bradykinesia, and resting tremors. Neuropathologically, the disease is characterized by the loss of more than 70% of dopaminergic neurons of the substantia nigra with surviving dopaminergic neurons often containing cytoplasmic inclusions known as Lewy bodies and Lewy neurites (Giasson & Lee, 2003).

Despite intense investigation, little is known about PD pathogenesis. With most cases of PD appearing to be sporadic, many have pointed to environmental toxins as potential contributing agents (reviewed in Menegon et al. 1998). Hypothesized mechanisms by which these toxins contribute to PD pathogenesis include oxidative stress, proteosomal dysfunction, and mitochondrial dysfunction. Besides environmental causes however, the identification of genetic mutations associated with PD suggests a genetic component to PD pathology as well. Such mutations have provided insights into potential pathology mechanisms.

The first such mutation identified was an alanine to threonine transition at position 53 (A53T) of the alpha-synuclein (α-syn) protein (Polymeropoulos et al. 1997). Soon thereafter, a second mutation

associated with PD was found in the same α-syn gene that causes a proline substitution for alanine at position 30 (A30P) (Kruger et al. 1998). Both mutations cause autosomal-dominant PD. The α-syn protein was subsequently found to be the major component of Lewy bodies and Lewy neurites (Spillantini et al. 1997), and therefore appears to be central to PD pathogenesis. However, defining its role in PD pathogenesis has proven to be immensely difficult.

α-synuclein is a 140-amino acid protein belonging to a family of synuclein proteins that include β- and γ-synucleins. The function of these proteins are unknown, but the most well-characterized of the three, α-syn, appears to have a role in vesicle regulation. α-syn knockout mice generated so far show subtle changes in dopamine (DA) turnover, but otherwise display no major abnormalities. In one α-syn knockout mouse, striatal DA was about 18% lower and DA release following a paired neural stimulus was higher (Abeliovich et al. 2000). In another independently generated α-syn knockout mouse, the knockouts show severely reduced DA release after repeated and prolonged neural stimulation (Cabin et al. 2002). In addition, cultured neurons from this mouse line had a reduced number of synaptic vesicles, especially in the reserve pool. Data from these two mouse lines therefore suggest that α-syn may have a role in synaptic vesicle regulation. However, conclusions drawn from the α-syn knockout mice must be interpreted with caution as the other synuclein proteins (β- and γ-synuclein) may be able to compensate for the lost α-syn in these mice (Goedert 2001).

Nevertheless, further support for α-syn's role in vesicle regulation comes from biochemical studies of α-syn. α-syn is a natively unfolded protein that assumes an α-helical conformation when it associates with membranes (Davidson et al. 1998 and Eliezer et al. 2001), so it may be possible that α-syn regulates vesicles through direct interaction with vesicular membranes. Jenco et al also showed that α-syn can selectively inhibit phospholipase D2, which has a role in cytoskeletal regulation and endocytosis (Jenco et al. 1998). Additionally, α-syn appears to be expressed throughout the brain and localizes to the synaptic terminals, in association with synaptic vesicles, further implicating its role in direct regulation of synaptic vesicles (Clayton et al. 1999). So far, studies of α-syn's non-pathology-associated functions have not suggested an immediately obvious mechanism for its disease-associated role. Nevertheless, the non-disease-associated role of α-syn is still being investigated as understanding its normal function may eventually shed insight into its pathological function.

Because the α-syn knockout mice show no PD-like pathology and because familial α-syn mutations are dominant mutations, the PD-causing α-syn mutations are likely to be toxic gain-of-function mutations rather than loss-of-function mutations. Therefore, analysis of the mutant forms of α-syn may provide greater insight into α-syn-related PD pathology. Such studies have been done in vitro and in vivo yielding interesting results which have led to several hypotheses on PD pathology. Among these studies that have yielded great insights into PD pathology are those using transgenic animal models over-expressing the various forms of α-syn.

Researchers have produced at least eight different transgenic mouse lines that over-express α-synuclein under various promoters (reviewed in Lotharius & Brundin 2002). Unfortunately, none of the mouse models reproduced the key features of PD. While α-syn appeared to accumulate in the majority of these transgenics, in only one line did the α-syn aggregates have Lewy Body-like morphology. (Van der Putten et al., 2000, Giasson et al., 2002). Despite the LB-like aggregates in these lines however, there was no neuronal loss in those areas with LB-like aggregates except in the ventral roots (Van der Putten et al., 2000, Giasson et al., 2002). None of the α-syn transgenic mouse lines displayed selective DA neuronal loss, though some lines exhibited a loss of DA terminals in the striatum (Masliah et al., 2000, Richfield et al., 2002). And finally, while some of the mouse lines exhibited motor deficits, the motor deficits did not appear to arise from DA neuronal loss, but rather from such things as axonal degeneration in the spinal roots (Van der Putten et al 2000, Giasson et al 2002). Despite the fact that α-syn over-expression failed to accurately model PD in mice, it is interesting to note that in a few of these studies, the over-expression of the mutant forms of α-syn resulted in a more severe phenotype than the over-expression of wild-type α-syn (Giasson et al. 2002, Lee et al. 2002, and Richfield et al. 2002). Nevertheless, attempts in mice to model PD pathology by α-syn over-expression has been less than a complete success.

Surprisingly, attempts to model PD pathology by α-syn over-expression in *Drosophila* has been much more successful. Feany and Bender over-expressed wild-type and mutant forms of α-syn throughout the *Drosophila* brain with the neuronal-specific *elav* promoter (Feany & Bender 2000). The flies developed LB-like inclusions in 2-10% of neurons throughout the brain. This is similar to what is seen in PD as LB's are typically not only detected in the substantia nigra, but also in the motor nucleus of the vagus nerve, hypothalamus, nucleus basalis of Maynery, locus coeruleus, cerebral cortex, olfactory bulb, and the

autonomic nervous system (reviewed by Goedert 2001). Furthermore, Feany and Bender detected age-dependent loss of dorsomedial cluster DA neurons in their transgenic flies. And finally, the transgenic flies exhibited age-dependent climbing ability deficits.

Because the α-syn-expressing fruit fly model develops LB-like inclusions, exhibits selective and age-dependent loss of DA neurons, as well as motor deficits, it has become a prime model for PD. However, the utility of the model is somewhat limited as it is an invertebrate model of PD and α-syn pathology, and hence there could be major differences in α-syn toxicity between flies and humans. Perhaps the most important of these differences is that *Drosophila* do not have an α-syn homologue. Because fruit flies do not have an endogenous synuclein, the effects of many of the key proteins that mediate PD pathology through interactions with α-syn might not be found with this model. On the other hand, vertebrates express their own endogenous α-syn, so a vertebrate model of α-syn toxicity may be more useful for studying PD.

Attempts to model PD in mouse by α-syn over-expression have so far proven difficult, and yet, as previously discussed, there are strong reasons for establishing a vertebrate model of α-syn toxicity. The zebrafish may be an ideal model to investigate the mechanism of α-syn toxicity as zebrafish have the advantage of being amenable to genetic and *in vivo* small compound screens. Here, in order to obtain a vertebrate model of α-syn toxicity, I have generated transgenic zebrafish (*Danio rerio*) that express human α-synuclein.

Because α-syn is expressed in the brain (reviewed in Goedert 2001), I expressed human wild-type and the two mutant forms of α-syn, A30P and A53T, in zebrafish using the neuronal specific promoter HuC. HuC is the zebrafish homologue of the fruit fly elav gene (Park et al. 2000), the promoter of which was used to direct α-syn over-expression in *Drosophila* by Feany and Bender (Feany & Bender, 2000). The 1st set of transgenic fish express the wild-type, A30P, or A53T form of α-syn fused to green fluorescent protein (GFP) under the control of the HuC promoter. Expression of α-syn as a GFP fusion protein allows for easy *in vivo* assessment of transgene expression and localization and may allow for direct observation of the α-syn aggregation process (Fig 1, set 1).

However, because fusion of GFP to α-syn may interfere with α-syn toxicity, I also generated a 2nd set of transgenic fish that simply express the three different forms of α-syn under the control of the HuC promoter (Fig 1, set 2).

To ensure strong α-syn transgene expression, a 3rd set of transgenic fish were generated, over-expressing the α-syn transgene by the GAL4-UAS over-expression system. I sought to ensure α-syn over-expression as previous reports (Singleton et al. 2003) suggests that α-syn pathology can be induced by higher amounts of α-synuclein (Fig 1, set 3).

When initial analyses of a few of the transgenic fish expressing the GFP-α-syn fusion protein suggested that the HuC promoter was not properly directing transgene expression in all neurons, I generated a 4th set of transgenic fish that express the α-syn genes under the direction of the CMV viral promoter or the EF1α promoter. Both these promoters should drive α-syn transgene expression in all tissues (Fig 1, set 4).

Utilizing the unique advantages of zebrafish, these α-syn transgenic fish may be used to further elucidate α-syn pathology and how it contributes to PD. For example, because of the ease of their maintenance and large clutch numbers, the zebrafish model has the advantage of being a vertebrate model that is amenable to mutagenesis and drug screens. Using the α-syn transgenic fish in a mutagenesis screen, one may find other genes involved in α-syn and PD pathology. Alternatively, one may use the transgenic fish in a drug screen to test and find new therapeutics as well as identify environmental toxins that may contribute to PD. Therefore, this new transgenic fish model has much to tell us about the mechanisms of α-syn toxicity and PD pathology.

Methods:

Cloning of 1st set transgenesis constructs: The HuC-promoter was provided by Cheol-Hee Kim (Park et al. 2000) in the plasmid pBS-3.2KbHuCP-EGFP. The EGFP-α-syn bearing plasmids (pEGFP-wt-xsyn, pEGFP-xsyn-A30P, and pEGFP-xsyn-A53T) were provided by Bingwei Lu. According to Lu, these EGFP-α-syn plasmids were generated by PCR'ing the human wildtype, A30P, and A53T mutant forms of α-syn with the primers: 5'-GATCAGATCTATGGATGTATTCATGAAAGGACTTTC-3' and 5'-

GATCCTGCAGTTAGGCTTCAGGTTCGTAGTCTTG-3'. The PCR product was then cut with BglII and PstI and cloned into BglII/PstI digested pEGFP-C1 plasmid from Stratagene.

Two forms of the HuC promoter were generated by PCR amplification of the pBS-3.2KbHuCP-EGFP plasmid. The 1ATG-HuC form was generated by PCR with NheHuC5 (5'-AGCGCTAGCGAATTCACTAATTTGAATTTAAATG-3') and 1ATGHuC3 (5'-GCTACCGGTGTACAAAGATGATAGTGATCTAG-3') primers. The 2ATG-HuC form was generated by PCR with NheHuC5 and 2ATGHuC3 (5'-GCTACCGGTACCATTCTTGACGTACAAAGATG-3') primers. The NheHuC5 primer hybridizes to the 5' end of the HuC promoter and introduces an NheI site there while the 1ATGHuC3 and 2ATGHuC3 primers hybridize to the 3' end of the promoter and introduce an AgeI site there.

The two forms of the HuC promoter were PCR'ed with Roche's Expand High Fidelity PCR kit. The PCR rxn was thermocycled with one cycle of 94°C for 2 min, then ten thermocycles of 94°C for 10 seconds, 55°C for 30 seconds, and 72°C for 3 min, followed by a 72°C for 7 min extension cycle). The PCR reactions were then cut with AgeI and NheI, and then purified with Qiaquick PCR purification kit from Qiagen. The HuC promoter was then ligated into NheI/AgeI cut and phosphatase-treated pEGFP-wt-xsyn, pEGFP-xsyn-A30P, and pEGFP-xsyn-A53T plasmids. The ligated plasmids were then transformed into XL-1 Blue *E. coli* (Stratagene). The resulting plasmids were checked by restriction digests and sequencing around the ligation sites.

Later, I removed the CMV promoters present in the 1ATG-HuC constructs by excising the CMV promoter with NheI and AseI. I then blunted the sticky ends with T4 DNA polymerase and ran the reaction out on agarose gel. I purified the vector band and ligated the vector to recircularize it. The ligations were transformed into XL-1 Blue *E. coli* (Stratagene). The resulting plasmids were then checked by restriction digests. The resultant plasmids were labeled pHuC-EGFP-αsyn(Pcmv-), pHuC-EGFP-A30P(Pcmv-), and pHuC-EGFP-A53T(Pcmv-).

Cloning of 2nd set transgenesis constructs: To generate the 2nd set transgenesis constructs I cut the 1st set transgenesis constructs (pHuC1ATGEαsyn, pHuC1ATGEA30P, pHuC1ATGEA53T, pHuC2ATGEαsyn, pHuC2ATGEA30P, and pHuC2ATGEA53T plasmids) with AgeI and BglII to excise

the EGFP gene. I ran the digest on an agarose gel and excised the vector band and purified the vector band out of the gel with Qiaquick Gel Purification Kit (Qiagen). I then blunted the sticky ends of the linearized plasmid with T4 DNA polymerase. I then ligated to recircularize the plasmid and transformed the ligation reaction into XL-1 Blue *E. coli* (Stratagene). Once cloned, the plasmids were checked by restriction digest analysis and sequence analysis around the ligation site. The plasmids were named p232(-gfp)αs1ATG, p234(-gfp)αs2ATG, p236(-gfp)AP1ATG, p238(-gfp)AP2ATG, p240(-gfp)AT1ATG, and p242(-gfp)AT2ATG (see Table 1).

Later, I removed the CMV promoters present in the 1ATG-HuC constructs by excising the CMV promoter with NheI and AseI. I then blunted the sticky ends with T4 DNA polymerase and ran the reaction out on agarose gel. I purified the vector band and ligated the vector to recircularize it. The ligations were transformed into XL-1 Blue *E. coli* (Stratagene). The resulting plasmids were then checked by restriction digests. The resultant plasmids were labeled pHuC-αsyn(Pcmv-), pHuC-A30P(Pcmv-), and pHuC-A53T(Pcmv-).

Cloning of 3rd set transgenesis constructs: The GAL4/VP16-UAS-E1b sequence was provided to us by R. Koster in the plasmid pBGal4VP16. To use the GAL4/VP16-UAS-E1b system to over-express a non-GFP tagged form of α-syn, I PCR'ed the GAL4/VP16-UAS-E1b sequence out of ApaI-linearized pBGalVP16 using the primers 183-5Age (5'-GCGTTACCGGTGATGAAGCTACTGTCTTCTATC-3') which introduces an AgeI site at the 5' end of the sequence for cloning purposes, and 183-3Ssp (5'-CCACCCAATATTGAATTCGTGTGGAGGAGCTC-3'), which introduces an SspI site at the 3' end of the sequence. The PCR was done using Roche's Expand High Fidelity PCR Kit. PCR reaction was first incubated at 94°C for 5 min, then thermocycled ten times at 94°C for 15 seconds, 53°C for 30 seconds, then 72°C for 90 seconds. Finally the reaction was incubated at 72°C for 5 minutes. After PCR, I cut the PCR product with AgeI and SspI and ran out the product on electrophoretic DNA gel. I purified the DNA out of the gel with Qiaquick gel purification kit. I ligated this PCR product into pHuC-EGFP-A30P(Pcmv-) vector that had been prepared as follows: I cut pHuC-EGFP-A30P(Pcmv-) with BglII and then phenol:chloroform extracted and ethanol precipitated the DNA. I then blunt-ended the vector by treating it with T4 DNA polymerase. I heat-killed the T4 DNA polymerase and then cut the vector with AgeI, which

essentially excised the EGFP gene out of the vector. I dephosphorylated the vector with calf-intestinal phosphatase and then heat-killed the AgeI. I ran the DNA out on electrophoretic agarose gel and purified the DNA with Qiaquick Gel purification kit.

To use the GAL4/VP16-UAS-E1b system to over-express a GFP-tagged form of α-syn, I PCR amplified the GAL4/VP16-UAS-E1b sequence from ApaI-linearized pBGalVP16. I used the primer 183-5Age (sequence above) and 183-3Age (5'-TTCATAACCGGTGAATTCGTGTGGAGGAGCTC-3'). I PCR'ed the sequence using Roche's Expand High Fidelity PCR Kit. The reaction was first incubated at 94°C for 5 min, then thermocycled five times 94°C for 30 seconds, 53°C for 45 seconds, then 72°C for 2 min. Then the reaction was thermocycled ten times 94°C for 30 seconds, 67°C for 45 seconds, then 72°C for 2 min. Finally the reaction was incubated at 72°C for 5 min. I purified the PCR product with Qiaquick PCR Purification Kit and then cut the product with AgeI. I ran out the reaction on electrophoretic agarose gel and gel purified the band with Qiaquick Gel Purification Kit. I ligated this GAL4/VP16-UAS-E1b sequence into pHuC-EGFP-A30P(Pcmv-) (plasmid containing 1ATG-HuC directing expression of A30P mutant αsyn fused to GFP) that had been cut with AgeI and de-phosphatased.

After ligation, the ligation reactions were transformed into TOP10 *E. coli*. The resultant plasmids were checked by restriction digests and sequence analysis. The plasmid constructs were labeled pHuC-281GAL-A30P and pHuC-281GAL-GFPA30P.

I then cloned the transgenes contained in these plasmids to the pTol2000 vector. I cut pHuC-281GAL-A30P and pHuC-281GAL-GFPA40P with ApaLI and MluI. I heat-inactivated the enzymes and blunted the plasmid with T4 DNA polymerase. I ran out the reaction on electrophoresis gel to separate the plasmid backbone from the transgene insert. I excised the transgene insert out of the gel and purified it out with Qiaquick Gel Extraction Kit. I then ligated this insert into pTol2000 that had been cut with NdeI and blunt-ended with T4 DNA polymerase. After ligation, I transformed the construct into TOP10 *E. Coli*. Plasmid clones were checked by restriction digest and sequence analysis around the ligation sites. The resultant plasmids were labeled pT-281GAL-GFPA30P and pT-281GAL-A30P.

Cloning of 4th set transgenesis constructs: To direct A53T mutant α-syn expression under the CMV promoter, I replaced the XIG promoter in pT2KXIG with the CMV promoter (P_{CMV}). To do this, I

prepared P_{CMV} DNA from pEGFP-N1 (Stratagene) by cutting the plasmid with ApaLI. I heat-inactivated the restriction enzyme and blunt-ended the ApaLI sticky ends with T4 DNA polymerase. I then ethanol-precipitated the DNA and cut the plasmid with SalI. I heat-inactivated the digest and ran the digest out on electrophoretic agarose gel. I excised the band corresponding to P_{CMV} and gel-purified it with Qiaguick Gel Purification Kit. To prepare the vector, I cut pT2KXIG with XhoI and then heat-inactivated the enzyme. I then blunt-ended the XhoI sticky end with T4 DNA polymerase, and then heat-inactivated the polymerase. I ethanol-precipitated the reaction and cut the vector plasmid with SalI. I heat inactivated the digest and treated the plasmid with calf-intestinal phosphatase. I ran the reaction out on agarose gel and purified the DNA from the gel with Qiaquick Gel Purification Kit. I then ligated the P_{CMV} DNA into the pT2KXIG vector. The ligation was transformed into TOP10 *E. coli*, and clones were screened and confirmed by restriction digest and sequence analysis. The resultant plasmid was labeled pT2KCMV.

I then cloned the A53T-mutant form of α-syn into pT2KXIG and pT2KCMV. The pT2KXIG plasmid was used to direct expression of the α-syn transgene under the EF1α promoter. The insert was prepared by cutting out the α-syn(A53T) mutant gene from pEGFP-xsyn-A53T with MluI. I heat-inactivated the digest and blunt-ended the sticky ends with T4 DNA polymerase. I ethanol-precipitated the DNA and cut it with BglII. After heat-inactivating the BglII digest, I ran out the reaction on a DNA gel and excised the band corresponding to the size of the α-syn(A53T) mutant gene. I purified this insert DNA from the gel with Qiaquick Gel Extraction Kit. To prepare the pT2KXIG and pT2KCMV vectors, I cut them first with ClaI. I heat-inactivated the digest and blunt-ended the ClaI sticky ends with T4 DNA polymerase. I ethanol precipitated the vector and cut it with BamHI, which would result in the excision of EGFP from the vector. I ran out the vector digest on agarose gel and purified the excised vector band with Qiaquick Gel Purification Kit. I then ligated the insert into both pT2KXIG and pT2KCMV vectors. I transformed the ligation into TOP10 *E. coli*, and checked the clones by restriction digest and sequence analysis. The resulting plasmids were labeled pT2KCMV-A53T and pT2KXIG-A53T.

Generating transgenic G_0 zebrafish: To generate transgenic zebrafish, I linearized the transgenesis plasmid construct and purified the plasmid by phenol:chloroform extraction followed by

ethanol purification. The plasmids were injected into 0-1 hour old zebrafish embryos. By about three months of age, the embryos would have grown into adults, at which time they may then be screened.

Later in the project, I had discovered a better method for generating transgenic G_0 fish. We made use of a transposase system developed by Koichi Kawakami to promote transgene incorporation into the chromosomes (Kawakami et al. 1999). To use the system, I would clone the transgene into either the pTol2000 or pT2KXIG vectors that contain the transposable sequences. The resultant plasmid would then be injected as a circular plasmid (ie uncut) with Tol2 transposase mRNA into 0-1 hour old embryos. Tol2 mRNA was prepared by in vitro transcription from pCS-TP as follows: pCS-TP was cut with NotI. RNA was then transcribed from this linearized plasmid using mMESSAGE mMACHINE SP6 RNA Polymerase Kit (Ambion, Cat # 1340). I purified the mRNA product with a purification column (Boehringer, Quick Spin, Cat # 1274 015) and extracted it with phenol-choloroform. I ethanol-precipitated the RNA and resuspended in nuclease-free water to a final concentration of 250ng/μL. RNA was stored at -70°C.

The micro-injected embryos would then be raised to adulthood and screened for the transgene. At eight hours post-injection a few of the deformed embryos were selected out and used for an excision assay as described by Kawakami & Shima (1999). The excision assay determines whether the transposase has successfully transposed the transgene out of the vector. The assay was done with the primers BS1 (5'-AACAAAAGCTGGAGCTCCACCG-3') and TYR1 (5'-AAGGCTCTTGGATACGAGTACGCC-3').

Screening for α-syn transgenic founders: To screen for transgenic founders (G_0) fish, I raised the micro-injected embryos to adulthood. I then crossed these adults together in breeding groups and obtained eggs from the crosses.

If the micro-injected construct contained GFP, I raised the eggs to 2-3 days old and observed them under a fluorescence dissecting scope. If any of the embryos exhibited GFP fluorescence, I surmised that one of the adults in the breeding group was a transgenic germline founder.

If the micro-injected construct was a not a GFP containing construct, I raised the embryos to 1 week of age before digesting them in groups of 50 embryos with Proteinase K (150ng/ml Proteinase K in 0.1x TE buffer). Digestion reaction was incubated at 50°C overnight and then heat-inactivated at 98°C for 15 min. I then PCR'ed each digestion for the presence of the transgene. For the HuC-αsyn constructs (2nd

set transgenesis constructs), I used the primers HuC3B (5'-GAGATTTCCTGGCGAAGAC-3') and ASYN5B (5'-CTGCTCCTCCAACATTTGTC-3'). For the GAL4/VP16-UAS-E1b containing constructs (3rd set transgenesis constructs), I PCR'ed with the primers HuC3B and GAL5 (5'-GTTTTGGGAGAGTAGCGACAC-3'). For the CMV and EF1α constructs (4th set transgenesis constructs), I PCR'ed with the primers intrn3 (5'-GTAGAATATTTCTGCATATAAATTCTG-3') and ASYN5B.

For all samples, I also PCR'ed for Wnt5a, an endogenous zebrafish gene, as a positive control. The Wnt5a PCR was done with the primers Wnt5A-F (5'-CAGTTCTCACGTCTGCTACTTGCA-3') and Wnt5A-R (5'-ACTTCCGGCGTGTTGGAGAATTC-3'). I incubated the PCR reaction at 94°C for 5 minutes, then thermocycled through 94°C for 30 seconds, 52°C for 30 seconds, and then 72°C for 90 seconds forty-five times. After thermocycling, I incubated the reaction at 72°C for 5 min. I ran out the PCR on agarose gel and looked for PCR product. The presence of a PCR product indicated that the progeny came from a breeding group with a transgenic G_0 founder fish. The absence of a PCR product suggested that none of the parents in the breeding group were germline transgene carriers.

Once a breeding group of fish was identified to have at least one G_0 founder, I crossed each fish in that group to a non-transgenic wild-type fish and assessed their eggs for the presence of the transgene in the same manner described above. In this way, I would be able to identify the G_0 founder fish in the breeding group.

Propogation of GFP-tagged α-syn transgenic lines: Transgenic G_0 founder fish were bred to non-transgenic wild-type fish and the progeny embryos collected (see figure 2). The F1 embryos were observed under a fluorescence dissecting scope at day 2-3 of age. Non-GFP fluorescent embryos were discarded and only GFP embryos were raised. These F1 generation embryos were presumed to be all heterozygotes. These F1 were raised to adulthood and bred to each other. 25% of the resulting F2 embryos are wild-type, 50% are heterozygotes, and 25% are homozygotes. The wild-type fish were identified under the fluorescence microscope as non-GFP fluorescent embryos and were discarded. This left 66% of embryos as heterozygotes and 33% as homozygotes. After raising these embryos to adulthood, I identified each fish as a heterozygote or homozygote by first crossing each fish to a non-transgenic wild-type. I collected the

progeny and then determined whether the parent fish was a homozygote or heterozygote using one of two methods. The first method involved observing the embryos at 2-5 days of age under a fluorescence dissecting scope. If all embryos were GFP fluorescent, then the parent fish was presumed to be a homozygote. If only 50% of the embryos were GFP fluorescent, then the parent fish was presumed to be a heterozygote. Alternatively, the second method involved randomly selecting eight embryos of the resultant progeny and testing for the transgene in each by PCR. In brief, I would fix the embryos in 100% MeOH. The embryos could be stored at -20°C in MeOH. I then withdrew the MeOH and allowed residual MeOH to evaporate off. I then digested each embryo in 20μL 150ng/mL Proteinase K in 0.1x TE buffer at 50°C overnight. I heat-inactivated the Proteinase K digestions at 98°C for 15 minutes and then PCR'ed each sample for the transgene with HuC3B and EGFP5 (5'-CTTGCCGTAGGTGGCATC-3') primers. If all eight embryos carried the transgene, as indicated by PCR, the parent fish would be considered a homozygote. Otherwise, the parent fish would be considered a heterozygote. Subsequent generations were propogated by crossing homozygote to homozygote.

Propogation of non-GFP-tagged α-syn transgenic lines: Transgenic G_0 founder fish were bred to non-transgenic wild-type fish and the progeny embryos collected (see figure 2). All F1 embryos were raised to adulthood. The minority of F1 adults carrying the α-syn transgene were then identified by PCR on tail-clippings from the adults. To tail clip, I would anaesthetize the fish in tricaine and cut off 1/3rd to ½ of the caudal tail fin. I digested the tail fin in 50μL 150ng/mL Proteinase K in 0.1x TE buffer at 50°C overnight. I heat-inactivated the Proteinase K digests at 98°C for 15 minutes before PCR'ing each digest for the presence of the transgene with HuC3B and ASYN5B primers for the 2nd set transgenics, HuC3B and GAL5 primers for 3rd set transgenics, or intrn3 and ASYN5B primers for 4th set transgenics. The presence of a PCR product would indicate that the fish is a transgenic heterozygote. Once the heterozygotes in the F1 generation are so identified, I would cross heterozygote F1's to each other to obtain the F2 generation. All of these F2 fish would be raised to adulthood. This F2 generation would by 25% wild-type, 50% heterozygotes, and 25% homozygotes. To differentiate wild-types from the transgenic fish, I would tail-clip the F2 adults and PCR for the transgene in the same way I did to differentiate the F1 heterozygotes. I

would discard the F2 wild-types and continue to differentiate between the heterozygotes and homozygotes in the remaining F2's by crossing each fish to a wild-type fish.

I then randomly selected eight embryos of the resultant progeny and tested each for the transgene by PCR. In brief, I fixed the embryos in 100% MeOH. The embryos could be stored at -20°C in MeOH. I then withdrew the MeOH and allowed residual MeOH to evaporate off. I then digested each embryo in 20μL 150ng/mL Proteinase K in 0.1x TE buffer at 50°C overnight. I heat-inactivated the Proteinase K digestions at 98°C for 15 minutes and then PCR'ed each sample for the transgene with HuC3B and ASYN5B primers. If all eight embryos carried the transgene, as indicated by PCR, the parent fish would be considered a homozygote. Otherwise, the parent fish would be considered a heterozygote. Subsequent generations were propogated by crossing homozygote to homozygote.

Assessing amount of α-syn expressed: I assessed the amount of α-syn expressed either in fry or adults. To extract proteins from fry, I homogenized groups of 100 fry in 200μL of extraction buffer (20mM HEPES pH 7.4, 120mM NaCl, 5mM EDTA, 10% glycerol, protease inhibitor [1 tablet of Roche protease inhibitor/50mL solution]) in glass hand homogenizer (1mL, Kimble-Kontes, Cat # K885450-0020). To extract proteins from adult brains, I anaesthetized the adult fish in tricaine. I excised the brain from the fish and rinsed it briefly in PBS before homogenizing it in 150μL of extraction buffer in glass hand homogenizer. After homogenization, the sample was cloudy white.

I then added 150μL of 8M urea to the homogenized tissue samples and vortexed vigorously 5-10 minutes. I added SDS to 2% and Triton X-100 to 1% and vortexed again for 5 minutes. I then added SDS sample buffer and incubated at 65°C for 5 minutes. I then spun the sample down and stored at -70°C.

Before running the protein sample on protein gel, I thawed it at room temp and heated at 65°C for 5 minutes. I spun down the samples at 4°C and loaded the supernatant onto 10-12% acrylamide protein gel. I electrophoresed the gel and transferred the protein in the gel to a nitrocellulose membrane with the XCell Blot II module from Invitrogen. Transfer was run at 70 V, for 1.5 hours. I blocked the membrane with 5% milk/PBS + 0.1% Tween-20 (PBS-Tween) at 4°C overnight. I probed the membrane in primary antibody diluted in blocking solution (5% milk/PBS-Tween) at room temperature for 1 hour. Primary antibodies

used include mouse anti-α-syn (BD Transduction Laboratories, Clone 42, Cat # S63320-050) used at 1/500 dilution and mouse anti-β-actin (Sigma, Cat # A5441) used at 1/5000 dilution.

I washed the membrane 2x for a few seconds, then once for 15 min, and then twice for 5 min all in PBS-Tween. I then hybridized the secondary antibodies onto the membraneat 4°C overnight. Secondary antibodies used include peroxidase-conjugated anti-mouse antibody (Amersham Pharmacia, Cat # NXA931) used at 1/5000 dilution or peroxidase-conjugated anti-rabbit antibody (Amersham Pharmacia, Cat # NA934V) used at 1/5000 dilution. I then developed the blot with ECL Plus Kit (Amersham Biosciences, Cat # RPN2132). I exposed different section of Kodak X – OMAT AR film (Eastman Kodak Co., Cat # 165 1454) to the blot for 10 min, 5 min, 2 min, and 1 min, then developed the film.

Sometimes I stripped the antibodies off the membrane in order to detect a different protein. In this case, I stripped the membrane in stripping buffer (100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl pH 6.7) at 50°C for 30 minutes. I then washed the membrane in PBS-Tween twice for a few seconds, then twice for 10 minutes each. I then blocked the membrane, hybridized 1° and 2° antibodies, and detected the proteins in the same manner as described.

Assessing where α-syn is expressed: For fish expressing the GFP-tagged α-syn, I was able to directly observe where the transgene was expressed by observing under the fluorescence dissecting scope. However, for finer analysis or to study the expression in non-GFP α-syn expressing lines, I would have to cryo-section the fish and do immunohistochemisty (IHC) on the sections.

To prepare embryos for cryo-sectioning, I would fix the embryos overnight in 4% PFA in PBS at 4°C. I would then wash three times in PBS for 10 minutes each (For long term storage, I would dehydrate the embryos by rinsing in 50% EtOH, then 70% EtOH. The embryos would be stored in 70% EtOH at -20°C. Before use, I would rehydrate the embryos by rinsing in 50% EtOH, then in PBS). I would then transfer the embryo into 15% sucrose/PBS for a few hours, then in 30%sucrose/PBS overnight at 4°C.

To prepare adult brains for cryo-sectioning, I would anaesthetize the adult fish in tricaine. I would then cut open the belly and chest of the fish to expose the heart, and then cut a blood vessel adjacent to the heart. I would stab the heart with a glass capillary needle pulled to a fine point and inject 25-35mL of cold PBS through the needle into the heart. I would then pump 25mL of cold 4% PFA/PBS. The fish was then

dunked into 4% PFA/PBS and allowed to fix O/N at 4°C. The brain was then extracted from the skull and washed 3 x 10 minutes in PBS. The brain may then be dehydrated into EtOH for storage in -20°C as described previously for embryos. Alternatively, the brain may be immediately equilibrated with 15% sucrose/PBS for a few hours, then with 30% sucrose/PBS at 4°C overnight.

To cut the tissue samples, the brain or embryo was frozen in liquid nitrogen in rubber tissue molds filled with Tissue-Tek OCT compound. The sections were then cut at 16-25μm thickness in cryostat.

To hybridize primary antibody onto the sections, I dried the slides at room temperature for over 30 minutes. I then washed the slides with 0.1% Triton X-100 in PBS (PBS-Triton) at room temperature for over thirty minutes, changing the solution once or twice. I then blocked the sections in 2% bovine serum albumin (BSA) in PBS-Triton at room temperature for at least one hour. I then hybridized the primary antibody diluted in PBST at 4°C overnight. Primary antibodies used include mouse anti-αsyn (BD Transduction Laboratories, Clone 42, Cat # S63320-050) used at 1/250 dilution, rabbit anti-tyrosine hydroxylase (Chemicon, Cat # AB152) used at 1/200 dilution, rabbit anti-GFP (Novus Biologicals, Cat # NB 600-308, or AbCam, Cat # AB6556) used at 1/750 dilution, and mouse anti-ubiquitin (Santa Cruz Biotechnology Inc., Cat # sc-8017) used at 1/1000 dilution.

To hybridize the secondary antibodies, I washed the slides with PBST 6 x 10 minutes. I then added the secondary antibodies diluted in PBST and incubated at room temperature for at least two hours. I washed the slides in the dark in PBS 3 x 10 minutes and then mounted with Dako Mounting Medium (DAKO Corp., Cat # S3023) and coverslip. Secondary antibodies used include AlexaFluor 488 goat anti-mouse IgG (Molecular Probes, Cat # A11001) used at 1/500 dilution and AlexaFluor 594 goat anti-rabbit IgG (Molecular Probes, Cat # A11012) used at 1/500 dilution.

Sections were then observed with fluorescence filters on a Zeiss light microscope.

Results:

Cloning. We obtained the HuC-promoter-containing plasmid, pBS-3.2KbHuCP-EGFP, from Cheol-Hee Kim (Park et al. 2000). He recommended that we clone our gene into the plasmid's NcoI site located at the 3' end of the HuC promoter. However, the EGFP-α-syn genes we obtained from Bingwei Lu contained a NcoI site at the α-syn ATG start codon. This meant that I could not simply use NcoI for

cloning the EGFP-α-syn gene to the HuC promoter, since cutting the EGFP-α-syn gene with NcoI would have resulted in the separation of the EGFP gene fragment from the α-syn gene.

Because I could not use the NcoI site, I decided to PCR out the HuC promoter from pBS-3.2KbHuC-EGFP with primers designed to introduce a NheI site to the 5'end and an AgeI site to the 3' end of HuC. These NheI and AgeI sites can then be used to insert the PCR'ed promoter product into the plasmids carrying the EGFP-α-syn genes; pEGFP-wt-xsyn, pEGFP-xsyn-A30P, and pEGFP-xsyn-A53T.

However, another complicating issue arose as I began to plan the PCR. With sequence analysis of the 3' end of the HuC promoter, I discovered that cloning at the suggested NcoI site would result in a four amino acid addition to the gene product's N-terminus since there was another ATG about twelve nucleotides upstream of the ATG contained in the NcoI site. While the addition of these four amino acids to EGFP-α-syn would likely not have a significant impact on the protein, I thought it best to remove these four amino acids. Yet, I was not sure whether these twelve nucleotides were essential to proper HuC expression. Therefore, I was unsure whether their removal was prudent.

Eventually, I decided to PCR amplify two forms of the HuC promoter. One form of the HuC promoter, which I labeled "2ATG-HuC", would preserve the twelve nucleotides. The other form of the HuC promoter, which I labeled "1ATG-HuC", would no longer have the twelve nucleotides so that genes expressed under it would not have the additional amino acids attached. The two forms of the promoter were then cloned into pEGFP-wt-xsyn, pEGFP-xsyn-A30P, and pEGFP-xsyn-A53T plasmids, immediately upstream of the EGFP-α-syn gene so as to direct its expression. The resultant plasmids carrying the 1ATG-HuC were labeled pHuC1ATGEαsyn, pHuC1ATGEA30P, and pHuC1ATGEA53T, depending upon which α-syn form they carried. In similar fashion, the resultant plasmids carrying the 2ATG-HuC were labeled pHuC2ATGEαsyn, pHuC2ATGEA30P, and pHuC2ATGEA53T. The plasmids were checked by restriction digests before I sent them to be sequenced at the ligation sites.
Sequencing information confirmed proper ligation. For some reason though, the 1ATG-HuC promoters had six additional nucleotides before the translation start ATG codon. Whether the addition of these six nucleotides would alter the HuC promoter's activity remains to be determined.

I then proceeded to clone the 2nd set of transgenesis constructs which would mediate HuC-directed expression of non-GFP-tagged α-syn. We needed this 2nd set of transgenesis constructs because the fusion of GFP to α-syn may interfere with α-syn's activity.

To generate the 2nd set of transgenesis constructs, I simply took the 1st set of transgenesis constructs and excised out the EGFP gene. Restriction digests and sequencing results at the ligation sites again confirmed that the procedure had gone well. Sequencing confirmed that the cloning had gone well, with the 2ATG-HuC having the first ATG in-frame with the ATG of α-syn. The plasmids were named p232(-gfp)αs1ATG, p234(-gfp)αs2ATG, p236(-gfp)AP1ATG, p238(-gfp)AP2ATG, p240(-gfp)AT1ATG, and p242(-gfp)AT2ATG (see Table 1).\

Table 1: Name and contents of the 2nd set transgenesis constructs

Name	Construct
p232(-gfp)αs1ATG	1ATG-HuC directing expression of human wild-type αsyn
p234(-gfp)αs2ATG	2ATG-HuC directing expression of human wild-type αsyn
p236(-gfp)AP1ATG	1ATG-HuC directing expression of A30P mutant αsyn
p238(-gfp)AP2ATG	2ATG-HuC directing expression of A30P mutant αsyn
p240(-gfp)AT1ATG	1ATG-HuC directing expression of A53T mutant αsyn
p242(-gfp)AT2ATG	2ATG-HuC directing expression of A53T mutant αsyn

Once these constructs were made, they were linearized, purified, and then micro-injected into 0-1 hour old zebrafish embryos. We observed GFP expression in the muscle cells of fish transfected with the GFP-αsyn plasmids. Since transgene expression was directed by the HuC promoter, a neuronal-specific promoter, transgene expression in the muscle was not expected. We guessed that perhaps the exogenous transgene expression was due to the presence of the CMV promoter in the pEGFP-C1 plasmid that these transgenesis constructs are derived from. So I excised these CMV promoters in the plasmids bearing the 1ATG-HuC's by cutting the CMV promoter out with AseI/NheI and then recircularizing the plasmids. Only the 1ATG-HuC constructs were selected because the transient transgene expression from 1ATG-HuC and 2ATG-HuC constructs appeared to be equivalent. After cloning out the CMV promoter, the plasmids were labeled pHuC-EGFP-αsyn(Pcmv-), pHuC-EGFP-A30P(Pcmv-), pHuC-EGFP-A53T(Pcmv-), pHuC-

αsyn(Pcmv-), pHuC-A30P(Pcmv-), and pHuC-A53T(Pcmv-). These plasmids were then micro-injected into zebrafish embryos.

I then continued on to generate the 3rd set transgenesis constructs that utilize the GAL4/VP16-UAS-E1b system to over-express α-synuclein. These constructs were made by PCR amplifying the GAL4/VP16-UAS-E1b sequence out of a plasmid provided by R. Koster (Koster et al. 2001) and inserted into the pHuC-EGFP-A30P(Pcmv-) plasmid such that HuC now drives expression of GAL4 and the E1b promoter drives the expression of α-syn (A30P) or GFP-tagged α-syn(A30P) in the new plasmid. I then cloned out the new transgenesis construct from these plasmids and inserted it into the pTol2000 plasmid to use a newly developed transposase system for better transgenesis efficiency (Kawakami et al. 1999). The resultant plasmid was labeled pT-281GAL-A30P and pT-281GAL-GFPA30P, based on whether they directed expression of GFP-tagged α-syn or non-GFP-tagged α-syn. I have not yet cloned the GAL4/VP16-UAS-E1b sequence into the constructs directing the expression of the other forms of αsyn (ie. Wild-type and A53T mutant), but the same strategy may be applied to attain similar over-expression constructs for these other forms.

When I began to see that many of the transgenic lines were not expressing the transgene in all neurons, as assessed by immunohistochemistry, I decided to clone transgenesis constructs that would direct the expression of the α-syn transgenes under the CMV and EF1α promoters. These promoters should direct expression of the transgene in all cells of all tissues. The cloning was done in plasmids containing the transposable elements for Tol2. I only cloned the non-GFP-tagged A53T mutant form of α-syn under the P_{CMV} and EF1α, but the same method can be used to clone the other forms of α-syn under these promoters. These constructs made up the 4th set transgenesis constructs.

Since these 2nd, 3rd, and 4th set transgenesis constructs contained transposable sequences, they were injected uncut with Tol2 transposase mRNA generated by in vitro transcription. At 8 hours post-injection, a few of the deformed embryos were selected out and a PCR-based excision assay was done on them to determine whether the transposase had transposed the construct out of its vector. In all cases, the excision assay indicated that the transposase had worked. The other non-deformed embryos were raised to adulthood.

Screening. Of the fish injected with 1st and 2nd set transgenesis constructs, about 50% survived to adulthood. Of those that survived to adulthood, less than 2% of fish carried the transgene in their germline (Table 2). The observed transgenesis frequency was lower than seen in previous transgenesis experiments (Park et al. 2000).

Table 2: Numbers of fish screened and G_0 α-syn transgenic founders identified in fish screens on fish injected with 1st and 2nd set transgenesis constructs.

injected transgene	# of fish screened	# of founders identified	% founders
HuC→ GFP-asyn(wt)	178	4	2.25
HuC→ GFP-asyn(A30P)	288	4	1.39
HuC→ GFP-asyn(A53T)	142	3	2.11
HuC→ asyn	58	1	1.72
HuC→ asyn(A30P)	164	3	1.83
TOTAL	830	15	1.81

Zebrafish embryos injected with the constructs utilizing Kawakami's Tol2 transposase system exhibited much greater mortality as approximately 20% of the embryos survived to adulthood. However, the frequency of germline transmission in the surviving adults was significantly greater with ~15% of them being germ-line founders.

Line Propogation. 1st set transgenesis constructs: Transgenic lines generated from the 1st set transgenesis constructs (GFP-tagged α-syn) were named asyn-1, asyn-2, asyn-3, asyn-4, A53T-1, A53T-2, A53T-3, A30P-1, A30P-2, A30P-3, and A30P-4, depending on the α-syn type being expressed.

Attempts to propagate these GFP-tagged α-syn expressing lines have been largely successful; nevertheless I was unable to propagate the lines asyn-2 and A53T-3. The G_0 founders of asyn-2 and A53T-3 did not breed well, so I was unable to attain an F1 generation.

The other lines were bred to homozygocity. Breeding to homozygocity was done by breeding the G_0 founder of the line to a non-transgenic wild-type fish. The F1 progeny embryos were collected and screened for GFP expression under a fluorescence dissecting scope. Non-fluorescent F1 embryos were

discarded and only the fluorescent ones were raised to adulthood. For most lines, the transgene germline transmission rate from the G_0 founder to the F1 generation ranged from 3% to 10% of F1 embryos. This was in line with previous trangenesis work (Park et al. 2000). F1s were then crossed to sibling F1s and the resultant F2 progeny were screened under the fluorescence dissecting scope to weed out the non-transgenic progeny. After selecting out the non-fluorescent F2s, the progeny were presumed to be $1/3^{rd}$ homozygous and $2/3^{rd}$ heterozygous. These F2 were raised to adulthood and the homozygotes were then identified by breeding each fish to a wild-type fish and determining what percentage of the resulting progeny were fluorescent. If all of the resultant progeny were fluorescent, the parental F2 fish was believed to be a homozygote. If only 50% of the resultant progeny were fluorescent, then the parental F2 fish was believed to be a heterozygote. Alternatively, instead of observing the progeny for fluorescence, eight progeny embryos were selected and digested for genomic DNA. I then did PCR on each embryo to detect the presence of the transgene. The presence of the transgene in all eight embryos indicated a homozygous parent. Otherwise, the parent was presumed to be a heterozygote. Once the homozygotes were identified in the F2 population, I crossed homozygotes to homozygotes to yield the subsequent populations of homozygotes only.

Using this method, I have successfully attained purely homozygote populations for asyn-1, asyn-3, A53T-2, A30P-1, and A30P-3 lines. I am currently trying to identify F2 homozygotes in asyn-4, A53T-1, and A30P-2 lines.

Interestingly the transgene genetics in the asyn-3 line is very odd. When F1 fish in this line are crossed to wild-type, about 99% of the resultant progeny are GFP fluorescent. I have yet to determine how or why these F1 heterozygous fish pass on the transgene like a homozygote.

2^{nd} set transgenesis constructs: The 2^{nd} set transgenic lines (α-syn not tagged with GFP) were designated with letters instead of numbers and were named asyn-A, A30P-A, A30P-B, and A30P-C.

Because the α-syn expressed in these lines is not tagged with a directly observable marker such as GFP, propagating these lines has been more involved. To do so, I would have to cross the G_0 founder to a wild-type fish and raise all the F1 progeny. I would not be able to weed out the non-transgenic F1s, as I had done with the 1^{st} set transgenics, because I can not tell which of the F1s are transgenic by fluorescence.

Therefore, to identify which F1s are heterozygotes, I would raise all F1s to adulthood and tail-clip each of them for genotyping. I would extract DNA from the tail-clip and use it to test for the presence of the transgene by PCR. With this method, I can tell whether a fish carries the trangene or not. F1 heterozygotes are then crossed to other F1 heterozygotes to yield the F2 generation. Again, I can not simply select out the wild-types in the F2 progeny by direct observation alone so I would raise all the F2 to adulthood. These F2 are expected to be 25% wild-type, 50% heterozygous, and 25% homozygous. I would tail-clip the F2s and thereby differentiate the wild-types, which would be discarded, from the heterozygotes and homozygotes.

Unfortunately, I can not use tail-clip genotyping to further differentiate the heterozygotes from the homozygotes, because the results only tell me whether the fish carries the transgene or not, and does not tell me exactly how many copies of the transgene it has. Consequently, to differentiate the heterozygous from the homozygous, I would breed an F2 fish to wild-type fish and randomly select out eight of the progeny. I would then PCR each progeny embryo for the transgene to determine whether the embryo was transgenic or not. If all eight of the progeny turn out to be transgenic, the F2 parent is probably a homozygote fish. Once F2 homozygotes are so identified, I would cross them together to attain pure homozygote populations. The transgenic line would then be maintained in homozygote populations.

Unfortunately, I have yet to even find transgenic F1s for lines asyn-A and A30P-C. The G_0 founders for these lines have not yielded many eggs lately and therefore may soon be lost. For line A30P-A, I have attained an F2 generation and will soon screen these F2s for homozygotes. For line A30P-B, I have identified one male and one female transgenic F1 fish, but I have not been able to breed the female. Therefore, I bred the F1 male to non-transgenic wild-type fish to attain the F2 population, which are expected to be 50% wild-type and 50% heterozygous. These will soon be screened for heterozygotes.

3^{rd} set transgenesis constructs: Transgenic lines generated with the 3^{rd} set transgenesis constructs were labeled GALA30P-A, GALA30P-B, and GALA30P-C. The method for propagating these lines is exactly the same as the method used for propagating the 2^{nd} set transgenic lines. Work in propagating these lines has only just begun as so far, I have only bred the G_0 founders to wild-type fish to attain the F1 population. These F1 populations have not yet been screened for transgenic heterozygotes.

Also, I have not yet screened embryos injected with pT-281GAL-GFPA30P plasmid, which should over-express the GFP-tagged A30P mutant form of α-syn, for G_0 founders.

4th set transgenesis constructs: I have identified only one transgenic line generated with the 4th set transgenesis construct pT2KCMV-A53T, and have named this line CMVA53T-A. These fish carry the transgene whereby the P_{CMV} drives the expression of untagged A53T mutant form of α-syn. Propagating this line involves the same propagation method used for the 2nd set transgenic lines. So far, I have only crossed the CMVA53T-A G_0 founder to non-transgenic wild-type fish and have raised the F1 progeny to adulthood. These F1s are now ready for sceening.

I have not yet screened the fish that were injected with 4th set transgenesis construct pT2KXIG-A53T, which would drive expression of the A53T form of α-syn from the EF1α promoter. Embyros have been micro-injected, but resulting adults have not yet been screened for G_0 founders.

Direct observation of transgene expression in 1st set transgenics. Because the 1st set transgenic lines express a GFP-tagged α-syn, I was able to follow transgene expression in the first several weeks of the zebrafishes' life, by simply looking at the embryo or fry (juvenile fish) under a fluorescence microscope. Up until two weeks of age, the tissues of the young zebrafish are transparent, which facilitates GFP detection. But after two weeks of age, the tissues of the growing fish attain pigments and become progressively less transparent, making GFP detection more difficult by these direct observation methods.

I photographed F1 heterozygotes at 1.5 days, 1 week, and 3 weeks of age under the GFP-stereomicroscope (Fig 3A). In non-transgenic fry, only the yolk-sac showed some degree of fluorescence under the GFP filters of the microscope. This fluorescence is not due to the presence of GFP, but other auto-fluorescent factors in the yolk sac. Indeed, the yolk-sac auto-fluorescence has been observed in all lines in the lab, transgenic and non-transgenic.

In addition to photographing developing heterozygotes at the juvenile stages of life, I also photographed the whole brain of adult fish from a few of the transgenic lines (Fig 3B). This was done by anaesthetizing the adult fish in tricaine and then excising the brain from the skull. The brain was then washed a few times in PBS and immediately photographed under fluorescence filters in a dissecting microscope.

Asyn-4 F1 heterozygote fry show relatively high levels of GFP expression, but the expression appears to be in many tissues during the first few days from birth (Fig 3A). Unexpectedly, GFP expression did not appear to be confined to the central nervous system. At 3 weeks of age, the level of GFP transgene expression is markedly reduced, but appears to be more localized to nervous tissue. The reduction in transgene expression appears to continue on into adulthood as the adult brain from this fish show only a slightly greater fluorescence than non-transgenic wild-type adult brain (Fig 3B)

At 1.5 days of age, A30P-1 F1 heterozygotes show very limited GFP transgene expression (Fig 3A). But transgene expression picks up by 1 week of age, when GFP signal can be clearly seen localized to CNS tissue. The GFP signal is even easier to see at 3 weeks of age. However, while the GFP signal appears to be present in most of the CNS tissue, it is strangely absent in the telencephalic region of the brain. The lack of complete GFP expression throughout the brain was also evident when I looked at the adult brain under the GFP dissecting scope (Fig 3B). While the adult brain definitely exhibited more fluorescence than the wild-type brain, the fluorescence signal did not appear uniform, as some parts of the brain showed greater fluorescence than other parts.

A30P-2 F1 heterozygotes also did not show complete CNS expression of the transgene (Fig 3A). At 1.5 days of age, transgene expression appeared to be localized to the extreme anterior end of the brain and the spinal cord. GFP expression spread, however, as by 1 week of age the transgene expression is seen in the telencephalon and the leading edges of the tectum. However, expression in the hindbrain was limited. The expression level of the GFP appears the highest of all 1st set transgenic lines. This is true even in the adults (Fig 3B).

A53T-2 F1 heterozygotes show relatively weak GFP expression at 1.5 days of age (Fig 3A). The expression appears to increase a bit by 1 week of age, and peters out by 3 weeks of age. I have not looked at the adult brain of the fish by direct GFP stereomicroscope observation.

I have also observed the GFP fluorescence in other lines, but not in as many multiple time points as those lines mentioned through Figure 3 (asyn-4, A30P1, A30P2, and A53T2). Asyn-1 F1 heterozygotes express the transgene in many tissues at day 1.5, much like asyn-4 heterozygotes. A53T-1 F1 heterozygotes show GFP expression at day 1.5 that all disappears by 3 weeks of age. Lines asyn-3, A30P-3, andA30P-4 have not yet been observed under the GFP dissecting scope.

Western blot determination of amount of α-syn transgene expressed. The results from direct observation of GFP fluorescence suggested great variability in transgene expression, both in amount and localization. To first assess the amount of the α-syn transgene expressed in different lines I decided to extract proteins from the lines and use them for a Western blot to get a semi-quantitative assessment of the amount of α-syn each line expresses. Unfortunately, initial protein extraction protocols were unable to extract any α-syn from the tissue samples. However, after much trial-and-error, I eventually settled on a protocol that was able to consistently extract α-syn from the zebrafish brains.

I tried the protocol on adult brains of non-transgenic wild-type, asyn-1, asyn-3, asyn-4, A53T-2, A30P-1, A30P-2, and A30P-3 adult F1 heterozygous fish. I then ran out the extracted proteins on an SDS-acrylamide gel, and transferred the proteins to nitrocellulose membrane. I then probed the membrane with anti-α-syn antibody. After detection, I stripped the membrane of antibodies and re-probed it with anti-β-actin. The β-actin stain told me how consistent protein loading was (β-actin is not involved in α-syn pathology and so the levels of β-actin protein should be fairly consistent between the different fish).

As suspected, the transgene expression levels were highly varied between the different transgenic lines (Fig 4). A30P-2 appeared to have the most GFP-α-syn transgene, which falls in line with direct observations of these and other fish under the GFP dissecting scope. A30P-1 was the next best-expressing line, followed by asyn-3, A30P-3, A53T-2, and then by asyn-4.

Asyn-1 appeared to have no GFP-α-syn transgene expression. While I was able to see ample GFP expression in this line at day 1.5 of age by observation under a GFP dissecting scope, the expression of the transgene in this line may decrease markedly in adulthood.

Interestingly, the lines expressing the most α-syn, A30P-2 and A30P-1, also had smaller α-syn fragments in the Western blot. The Western blot band for the GFP-α-syn fusion protein expressed in the 1st set transgenic lines came out as expected close to 50KDa. However, in the A30P-2 and A30P-1 lines, there was a smaller band that was about 36 KDa. This smaller band is expected to be a degradation product of α-syn as described by Petrucelli et al., in experiments on α-syn expressed in fruit fly (2000).

Also of note, the GFP-α-syn transgene expressed in the A30P-1 line is smaller than the GFP-α-syn expressed in other transgenic lines. How this is so is yet to be clarified.

The β-actin stain showed that indeed the protein loading was fairly consistent. In the future, I plan to assess the transgene expression level in the adults of all other lines (including 2nd, 3rd, and 4th set transgenics). I may also use this method to determine the amount of transgene expressed in fry, as there may be up- or down-regulation of the protein with age.

Localization of α-syn transgene expression by immunohistochemistry. To assess the extent and localization of transgene expression in the adult brain, I cryo-sectioned adult brains of wild-type and of heterozygous F1s of lines asyn-3, A30P-1, and A30P-2. To do so, I first perfused the fish with PBS and 4% PFA/PBS to remove blood from the brain and fix it. I then excised the brain and sectioned it with a cryostat into 14-25μm sections. I then hybridized anti-α-syn and anti-TH antibodies to the sections. The anti-α-syn antibodies were then developed with green fluorescent secondary antibodies. The anti-TH antibodies were developed with red fluorescent secondary antibodies. The α-TH antibodies would hybridize to noradrenergic neurons and dopaminergic neurons, the neuronal types lost in PD. Therefore, what we hoped to see was the majority of DA neurons (ie. TH-positive neurons) containing α-syn aggregates suggestive of PD.

Immunohistochemistry (IHC) on asyn-3, A30P-1, and A30P-2 adult F1 heterozygotes showed that α-syn rarely co-localized with TH. This suggests that dopaminergic neurons rarely express α-syn by adulthood. However, other neurons, besides DA neurons, do express abundant amounts of α-syn. Several distinct neuronal clusters in the brains of the transgenic fish have many neurons that express α-syn. Nevertheless, as a whole, these α-syn-expressing neurons appear to be a relative minority of neurons.

I found no DA neurons with α-syn expression in both of the A30P-1 adult brains I processed for IHC. Other neurons in the brain of A30P-1 adults, however, do exhibit strong GFP-α-syn expression. Particular neuronal clusters in the brain contain an abundance of neurons with α-syn expressed in their cell bodies. Also, plenty of axonal processes also contained GFP-α-syn. However, such α-syn-containing cells made up just a small percentage of the neurons in the brain.

In the two A30P-2 adult brains I processed for IHC, only one DA neuron in each brain was found to contain α-syn in their cell bodies (Fig 5 A & B). In the first brain, the α-syn appears to be present in both cytoplasm and nucleus (Fig 5A). Interestingly, in the second brain, the DA neuron expressing α-syn

appears bloated and distended, suggestive of pathology (Fig 5B). Additionally, some of the α-syn present in this neurons appears to be focally concentrated in distinct regions of the cytoplasm, which may be suggestive of α-syn aggregation. Indeed, focal clustering of α-syn was also fairly commonly seen in axons. One DA neuron in an A30P-2 adult did not appear to have any α-syn in its cell body, but there appeared to be focal clusters of α-syn in its axon (Fig 5C). Similar α-syn clusterings in axonal processes were observed in several non-DA neurons of the A30P-2 brains (Fig 5D). Like with the A30P-1 adult brains, some neuronal clusters contained many neurons with GFP-α-syn in the cell bodies, however such neuronal clusters were in the minority. Nevertheless, the α-syn stains in many of these neurons has a grainy appearance, which suggests that some of the α-syn has aggregated rather than remain evenly distributed throughout the cytoplasm

In the single asyn-3 adult brain I processed for IHC, there were many more DA neurons that express α-syn (Fig 6). However, such α-syn-expressing DA neurons were in the minority, as most DA neurons did not exhibit detectable levels of α-syn. Moreover, these α-syn-expressing DA neurons were confined largely to the telencephalon. No such DA neurons were observed in more posterior portions of the brain. Among non-DA neurons, however, there were distinct neuronal clusters that express high levels of α-syn, similar to what was observed in the A30P-2 adult brains. The identity of these neurons are still unknown. Interestingly, the α-syn signal in these neurons also has a grainy appearance which again suggests that the α-syn is not evenly distributed throughout the cytoplasm but has aggregated into several clusters. Additionally, many neurons had focal clusterings of α-syn in their axons, also reminiscent of what was observed in A30P-2 adult brains.

Other lines have yet to be analyzed by IHC. Even data from the lines looked at so far is still rather preliminary until several adult brains have been analyzed. In the future, I hope to use other stains to determine whether α-syn is aggregated in the neurons and whether the neurons are dying. I also hope to apply stereologic counting techniques along with confocal microscopy on these sections to determine if the transgenic adult brains have reduced numbers of DA neurons as compared to non-transgenic control.

Discussion:

I have generated several lines of α-syn transgenic fish using different transgenesis constructs (Fig 1). I have begun to analyze some of the 1st set transgenic fish as screening and propogation of these lines have been much simpler due to the immediately detectable GFP portion of the transgene. The analysis of these 1st set transgenics have consisted of an assessment of each line's expression level of α-syn and a determination of which of their neurons contain α-syn. The second set transgenic fish, expressing non-GFP-tagged α-syn, will also soon be ready for similar assessments of transgene expression.

Preliminary IHC analysis of a few of the 1st set transgenic lines suggests that the HuC promoter used in the 1st set transgenesis constructs does not drive the α-syn transgene expression in all neurons. The inability of HuC to drive expression of the transgene in all neurons is not likely due to the omission of the 2nd ATG from the HuC promoter. The HuC promoter provided to our lab contained two ATG start codons at the 3' end of the gene. Dr. Park, the provider of the HuC promoter, suggested that we clone our transgene into the 2nd ATG of the promoter. However, doing so would mean the addition of four amino acids to the transgene product's N-terminus. On the other hand, cloning the transgene onto the 1st ATG instead of the 2nd may disrupt proper HuC activity. To address these concerns, I simply cloned the transgene into both ATG's. Whether the transgene was cloned into the 1st or second ATG, however, does not seem to matter as lines where the transgene was cloned into the 1st ATG (ex. A30P-1 and A30P-2) and a line where the transgene was cloned into the 2nd ATG (ex. asyn-3) all do not show pan-neuronal transgene expression.

Therefore that leaves the possibility that the HuC promoter might not drive α-syn expression in all neurons as expected simply because the HuC is not endogenously expressed in all neurons. Alternatively, the HuC promoter's inability to drive transgene expression is because pan-neuronal α-syn expression is extremely toxic to the zebrafish. If pan-neuronal α-syn expression is indeed toxic, my transgenic lines may be surviving only because they have suppressed HuC-driven pan-neuronal expression. In these lines, HuC may have integrated into the genome near suppressor sequences that inhibit HuC-driven α-syn expression in all neurons.

This second possibility of pan-neuronal α-syn expression being toxic may also explain the low transgenesis frequency. In previous transgenesis experiments, injection of the transgenesis construct into

zebrafish embryos results in about 3% of those embryos becoming G_0 transgenic founders (Park et al. 2000). Our attempts to make 1st set and 2nd set transgenics have yielded less than 2% of micro-injected embryos becoming transgenic founders (Table 2).

A few caveats to this second possibility is that in human PD, α-syn toxicity takes years to manifest as PD is a slowly progressing neurodegenerative disorder that typically afflicts patients in their 50's and 60's. Therefore, the thought that pan-neuronal α-syn expression is immediately fatal to developing embryos is suspect. Moreover, the fact that the α-syn transgene is expressed at very high levels in these fish could make the transgene embryonic lethal. Nevertheless, previous attempts to create α-syn over-expressing mice have generally been fairly successful (reviewed in Dauer & Przedborski, 2003). Moreover, these transgenic mouse models have exhibited α-syn-associated toxicities only at advanced ages, and not during early development. If α-syn over-expression is indeed lethal to embryonic development, such lethaility has not been observed in mice. However, Dauer & Przedborski have also noted that mice appear to be particularly resistant to α-syn toxicity (2003).

Yet a third possibility is that pan-neuronal α-syn expression is not neccessarily toxic, and that the fact that the first three lines analyzed by IHC did not show pan-neuronal expression has been simply a matter of bad luck on our part. The HuC-GFP-αsyn transgene in these lines may have simply intergrated in the genome near particular enhancer and/or suppressor sequences that alter normal HuC expression, resulting in HuC failing to drive pan-neuronal transgene expression.

To differentiate between the first possibility, that the HuC promoter we got does not normally drive transgene expression in all neurons, from the second possibility, that pan-neuronal α-syn expression is toxic, I will do an IHC analysis on HuC-EGFP fish. These HuC-EGFP fish were provided to us by Park, the same contributor who provided us with the HuC promoter. If the HuC promoter does not effectively drive EGFP transgene expression in all neurons, then the lack of α-syn expression in all neurons is simply a result of the HuC promoter's inability to do so. On the other hand, if the HuC promoter does effectively drive EGFP transgene expression in all neurons, then α-syn may indeed be toxic when expressed pan-neuronally.

I suspect that pan-neuronal expression of the α-syn transgene is immediately toxic to the fish because a whole brain excised from a HuC-EGFP adult fish (data not shown) appears uniformly GFP-

fluorescent, but a whole brain excised from an A30P-1 or A30P-2 adult fish displays mosaic GFP-fluorescence (Fig 3B). Nevertheless, a look at the whole brain under a GFP dissecting scope is only a gross assessment of the extent of HuC-driven expression in the brain. I will be more confident of my suspicions that pan-neuronal α-syn expression is toxic only after I have done the IHC analysis on cryo-sectioned HuC-EGFP adult brain. In the meantime, I will continue to hope that I have simply been unlucky in selecting to analyze transgene expression in those lines that do not have pan-neuronal expression, and that other transgenic lines will exhibit pan-neuronal transgene expression.

But, if it indeed turns out that the transgene can not be expressed in all neurons, then can these α-syn transgenic zebrafish fish accurately model PD? I had hoped to express the α-syn transgene in all neurons since in humans α-syn is expressed in most brain regions. However, transgene expression in DA neurons alone may be sufficient to accurately model PD. PD pathology results from the loss of the majority of DA neurons in the substantia nigra. Surviving DA neurons usually contain the α-syn aggregates known as Lewy Bodies, suggesting that it is α-syn's actions in DA neurons that make it responsible for PD pathology. In other words, even though α-syn is normally expressed throughout the brain, in PD, its toxic functions seems to be selectively activated in DA neurons. Therefore, as long as the α-syn transgenic zebrafish express α-syn in DA neurons, it may still prove to be an excellent model for PD.

Unfortunately, in the lines analyzed by IHC so far (asyn-3, A30P-1, and A30P-2), only a minority of DA neurons appear to express the α-syn transgene. I will continue to do IHC on cryo-sectioned brains of other transgenic lines in the hopes of identifying a line with the majority of its DA neurons expressing the α-syn transgene. However, if, as I suspect, pan-neuronal α-syn expression results in embryonic lethality, then I may be hard-pressed to find lines with DA neuron α-syn expression, especially when considering α-syn's selective toxicity for such neurons in PD.

Even if that were the case though, these α-syn transgenic fish may still be suitable for studying the general mechanisms of α-syn aggregation and toxicity. While certain qualities of the substantia nigra DA neurons make them particularly susceptible to α-syn toxicity, α-syn can exert toxic effects in other neuronal types as α-syn pathology is also seen in other disorders such as Alzheimer's disease and multiple system atrophy (Goedert, 2001). So while transgenic fish that do not have α-syn expressing DA neurons could not be used to study what makes DA neurons particularly susceptible to α-syn toxicity, they may shed light on

the general mechanisms of α-syn toxicity. Understanding these general mechanisms of α-syn toxicity will be relevant to understanding α-syn's role in PD.

With this in mind, it is encouraging that even though the 1st set transgenic lines studied so far show few α-syn-expressing DA neurons, the neurons that do express α-syn show potential signs of α-syn pathology. In the two highest-expressing α-syn lines, A30P-1 and A30P-2, Western Blot analysis revealed that the α-syn transgene was being processed into smaller fragments. Petrucelli et al. has observed similar degradation in neuroblastoma cell lines over-expressing α-syn (2002). These α-syn degradation products suggest that the neurons may be responding to toxic levels of α-syn.

Furthermore, in asyn-3 and A30P-2 adult brains, I observed several Lewy neurite-like concentrations of α-syn in the axons of both DA and non-DA neurons (Fig 5D). Normally, α-syn is localized to the synapses. Many hypothesize that under pathological conditions, α-syn first oligomerizes at the synapse and is then transported through the axons to the cell body. Along the way, the α-syn further aggregates into Lewy neurites. When those Lewy neurites finally make it to the cell body, they complete the aggregation process by forming Lewy bodies. Therefore, the existence of focal concentrations of α-syn in the axons suggestive of Lewy neurites hints at potential α-syn pathology. Indeed, I had expected α-syn-expressing DA neurons to have α-syn present largely in their axons and processes. However, in all the transgenic lines, DA neuronal processes with α-syn were rare. Nevertheless, since some of these transgenic lines like A30P-2 do show signs, like Lewy neurite-like structures, indicative of α-syn pathology, they may still be useful to the study of α-syn pathology. Hopefully though, we will soon identify lines with the majority of its neurons expressing α-syn.

Regardless of whether the lines I find do or do not have many α-syn-expressing DA neurons, I must first prove that the many α-syn focal inclusions I observe (Fig 5B, C, D) are indeed α-syn aggregates. To do so, I may stain the neurons with thioflavin S, a marker of fibrillar aggregates (Manning-Bog et al. 2002). Alternatively, I may stain the brain sections for ubiquitin. Lewy Bodies, after existing for some time in the cytoplasm, become ubiquitinated as the cell tries to cope with the large aggregate (Giasson & Lee, 2003). Therefore, co-staining neurons for ubiquitin along with α-syn, may indicate whether the α-syn is in a Lewy-body-like form. Nevertheless, the preferred method for demonstrating that the α-syn is aggregated is by staining with thioflavin S.

Also, to show that the transgenic model may be used to study α-syn pathology, the model must demonstrate some neuronal loss as a result of α-syn expression. Indeed, the lack of α-syn-expressing DA neurons in A30P-1, A30P-2, and asyn-1 may be due to the loss of such neurons before I analyzed them by IHC. However, while I did not actually count the number of DA neurons in these fish, by general observation, they appeared to have a normal complement of DA neurons. Therefore I think it unlikely that the α-syn-expressing all died by the time of IHC analysis. Nevertheless, testing these lines for neuronal loss may prove me wrong. To test for neuronal loss, I may stain degenerating neurons with fluorojade B or silver stain. While fluorojade B has been previously used to stain degenerating neurons (Bianco et al. 2002), I have not had much luck with the reagent and have not been able to stain my cryosections properly with it. Alternatively, I may use silver stain which has also been used to stain degenerating neurons resulting from α-syn-related pathologies (Manning-Bog et al. 2003). Another possible method to demonstrate neuronal loss is by counting neurons at different ages. Counting would be a time-consuming process and so a more likely alternative would be to use the optical fractionator method, a stereologic technique, to obtain a statisitical estimate of the number of neurons (Keuker et al. 2001). However, the optical fractionator method requires more equipment, namely a confocal microscope and the appropriate computer programs. If α-syn-expressing neuronal populations decrease with age, it should become evident by counting or optical fractionation. Regardless of which method I use, showing neuronal loss would be important to demonstrating the validity of the α-syn transgenic fish for the study of α-syn pathology.

In summary, to prove these transgenic lines suitable for the study of α-syn pathology I must demonstrate α-syn aggregation as well as neuronal degeneration. Ideally, the transgenic line would express α-syn in DA neurons and DA neurons would exhibit the brunt of the pathology. Such a model could be used specifically for the study of α-syn's role in PD pathology. However, for such a model, I must first find a transgenic line that expresses α-syn in its DA neurons. Of the three lines studied so far, A30P-1, A30P-2, and asyn-3, only a minority of DA neurons showed much α-syn expression. I have yet to conduct an IHC analysis of the other transgenic lines.

Clearly, I have much work ahead of me yet in this project. I plan to characterize normal HuC expression in the HuC-EGFP fish by IHC. Characterization of theHuC expression in these HuC-EGFP fish will tell me whether I can expect the HuC to drive transgene expression in DA neurons. I also plan to

secure the 2nd, 3rd, and 4th set transgenic lines. With many of these lines, I am still in the process of finding F1 heterozygote progeny from the G_0 founder. With a few of these lines, I have yet to even screen the micro-injected fish for G_0 founders. Once the lines are secured, I plan to identify the lines with the highest α-syn expression by Western blot analysis. I will then determine where the lines express the transgene by IHC. Based on Western blot and IHC analyses, I will select the highest pan-neuronal α-syn-expressing line for further analysis. While lines expressing lower levels of α-syn may be interesting and studied in the future, the higher expressing lines are of greater interest because of the association between α-syn pathology and high levels of α-syn expression (Singleton et al. 2003). Also, lines that express high levels of α-syn early, but little to no α-syn in later adulthood may also be of interest in further studies as they may indicate whether neurons can recover from α-syn associated toxicities. Such a study would have deep implications for the development of a treatment for advanced PD. Therefore, lines with lower α-syn expression will be kept and maintained. However, for the immediate future, further analyses will be conducted only on those transgenic fish with the highest pan-neuronal expression.

Further analyses will include an assessment of α-syn aggregation by the methods mentioned previously. I will also determine whether neurons degenerate by the methods mentioned previously.

If, after these analyses, an α-syn transgenic line proves to display α-syn-related pathologies, I will compare the endogenous zebrafish α-syn expression with the transgene human α-syn expression. This comparison can be done by quantitative RT-PCR using zebrafish α-syn and human α-syn-specific primers on transgenic brain mRNA. Alternatively, I may quantify blot intensity on Western blots of transgenic brain proteins probed with zebrafish-specific and human-specific anti-α-syn antibodies. If the DA neurons show a high degree of α-syn-related pathologies I may also conduct assessment of the transgenics' locomotor ability with video-recording and tracking software (ex. DIAS).

I will then do a time-course analysis on transgenic fish of different ages to study when α-syn begins to aggregate, when neurons begin to die, and when locomotor defects begin to develop over the lifetime of the transgenic fish. Since PD is an age-related disease, I would hope that the transgenic model would similarly show an age-related pathology.

Finally, I will assess how different neurotoxins may enhance the disease phenotype. If the transgenic fish model is to be used for a genetic screen, it would be ideal if it developed a disease

phenotype at an earlier age. Neurotoxins may be used to induce an early disease phenotype. Also, in the case that I am unable to develop an α-syn transgenic model that displays pathology, these fish may show pathology under neurotoxin treatment. The presence of large amounts of α-syn in the neurons of these fish may sensitize these fish to the effects of PD-contributing toxins like MPTP and paraquat (Beal, 2001).

With such studies, I may soon develop α-syn transgenic lines that model many of the features of α-syn pathology and PD. Such a model could then be used to elucidate the pathology mechanisms by forward genetic screens or by a candidate gene approach. Or the model may be used to screen for potential therapeutic drugs that combat α-syn pathology. In these ways, an α-syn transgenic fish model of PD would be a great benefit to PD research.

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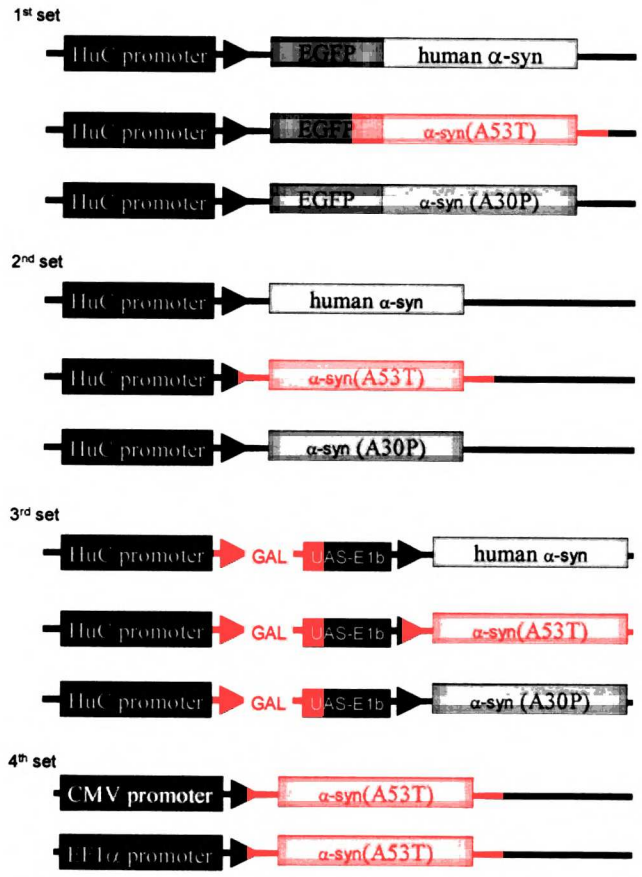
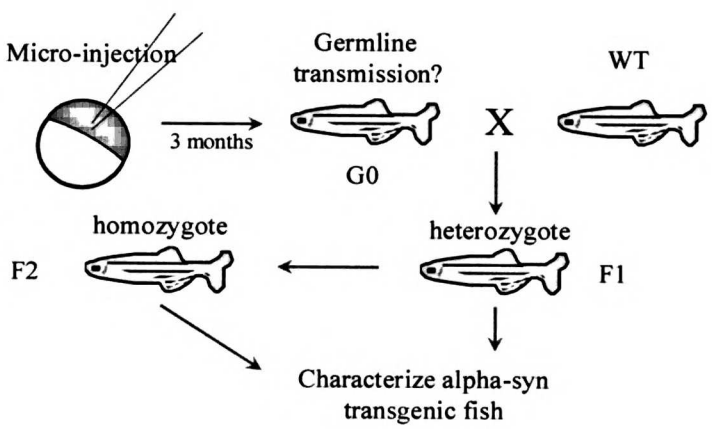


Figure 1 – Schematic of the DNA constructs used for transgenesis. In set 1, HuC drives expression of GFP-αsyn fusion proteins. In set 2, HuC drives expression of α-syn alone. In set 3, HuC drives expression of the GAL4 activation domian which in turn drives very strong expression of α-syn via the UAS-E1b promoter. In set 4, the α-syn (A53T) mutant is expressed by either the CMV or XIG promoters

Figure 2 - Creating α-syn transgenic fish. Transgenesis constructs are micro-injected into 0-1 hour old embryos. The embryos are raised and screened for G_0 germline founders. The G_0 founders are then crossed to wild-type fish to attain heterozygote F1 fish. Heterozygote F1 fish are then bred to each other to attain the F2 generation containing a mixture of wild-types, heterozygotes, and homozygotes. F1 and F2 fish are used to analyze the effects of α-synuclein.



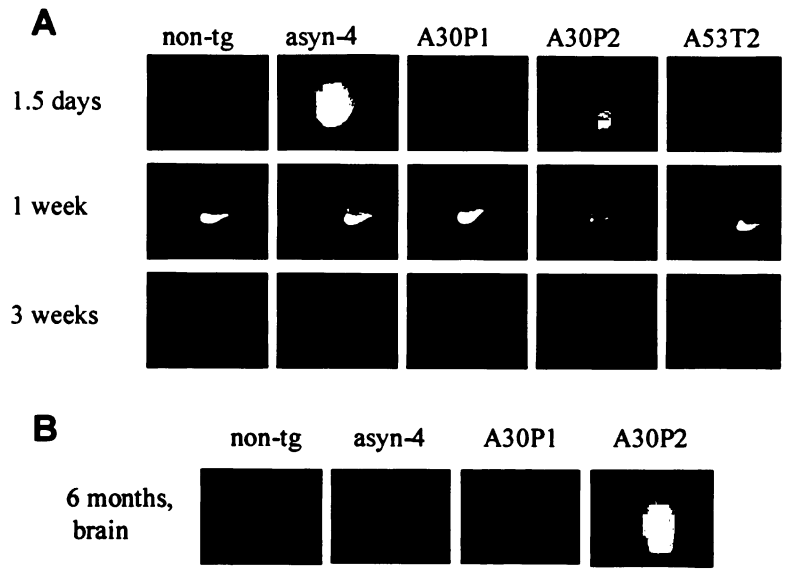


Figure 3 – Direct detection of GFP fluorescence in 1st set transgenic lines. Each line displays different GFP expression patterns. (A) F1 heterozygotes at 1.5 days, 1 week, and 3 weeks of age were photographed under a stereo-microscope with GFP fluorescence filters. (B) F1 heterozygote adults at 6 monts of age were anaesthetized and their brains extracted. The whole brains was then immediately photographed under a stereo-microscope with GFP fluorescence filters.

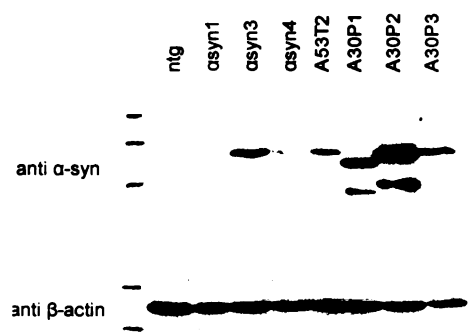


Figure 4 – Western Blot analysis of α-syn expression in some of the 1st set transgenic lines. After probing for α-syn, the same blot was stripped and re-probed for β-actin to assess the consistency of protein loading (bottom).

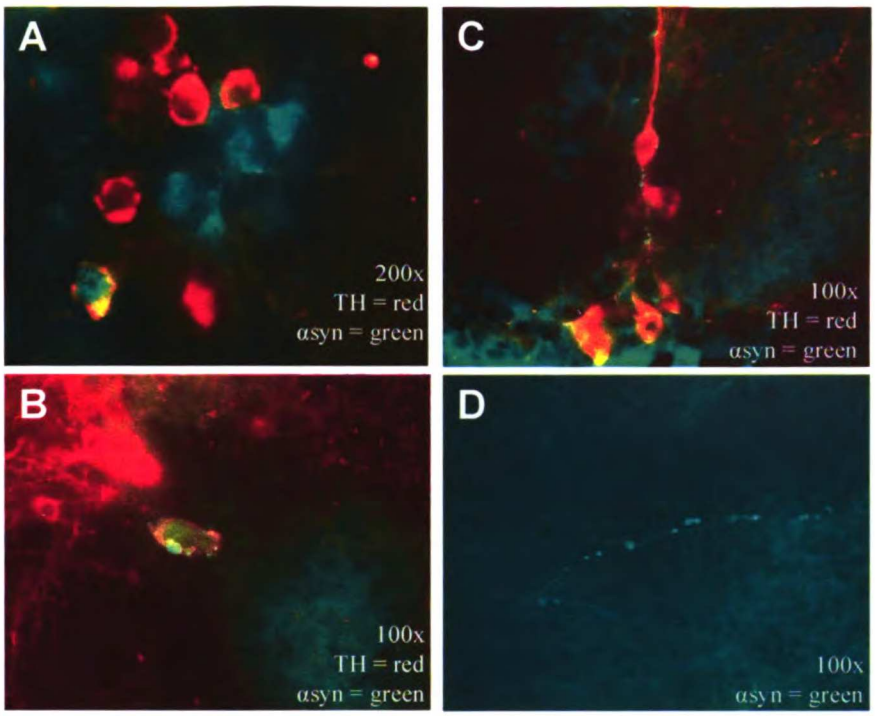


Figure 5 – Immunostaining results in A30P-2 heterozygous F1 adult brain. The only DA neurons found in two different A30P2 heterozygous adult brains that express α-syn in their cell bodies (A & B). TH labels the DA neurons and is stained red. α-syn is stained green. Note that in the first brain (A), the α-syn appears to be present in both cytoplasm and nucleus. In the second brain (B), the DA neuron expressing α-syn appears bloated and distended, suggestive of pathology. Additionally, some of the α-syn present in this neurons appears to be focally concentrated in distinct regions of the cytoplasm, suggestive of α-syn aggregation. Similar α-syn clusterings were observed in another DA neuron of the second A30P-2 fish, but not in the cell body (C). Instead, the α-syn clusters were in the axon of the DA neuron. Other non dopaminergic neurons in both A30P-2 adults showed α-syn clusters in their axons (D).

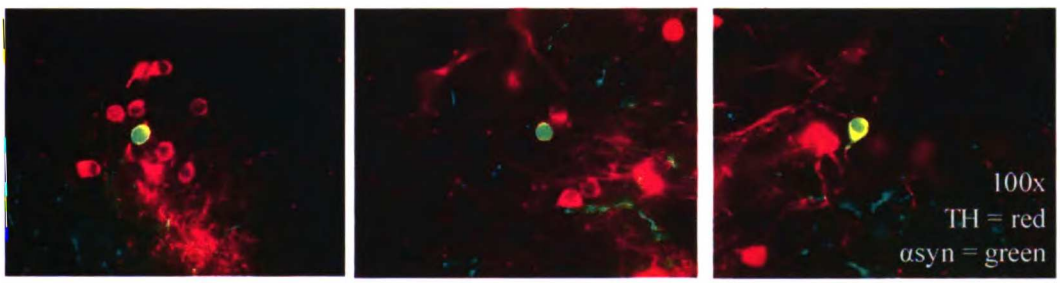
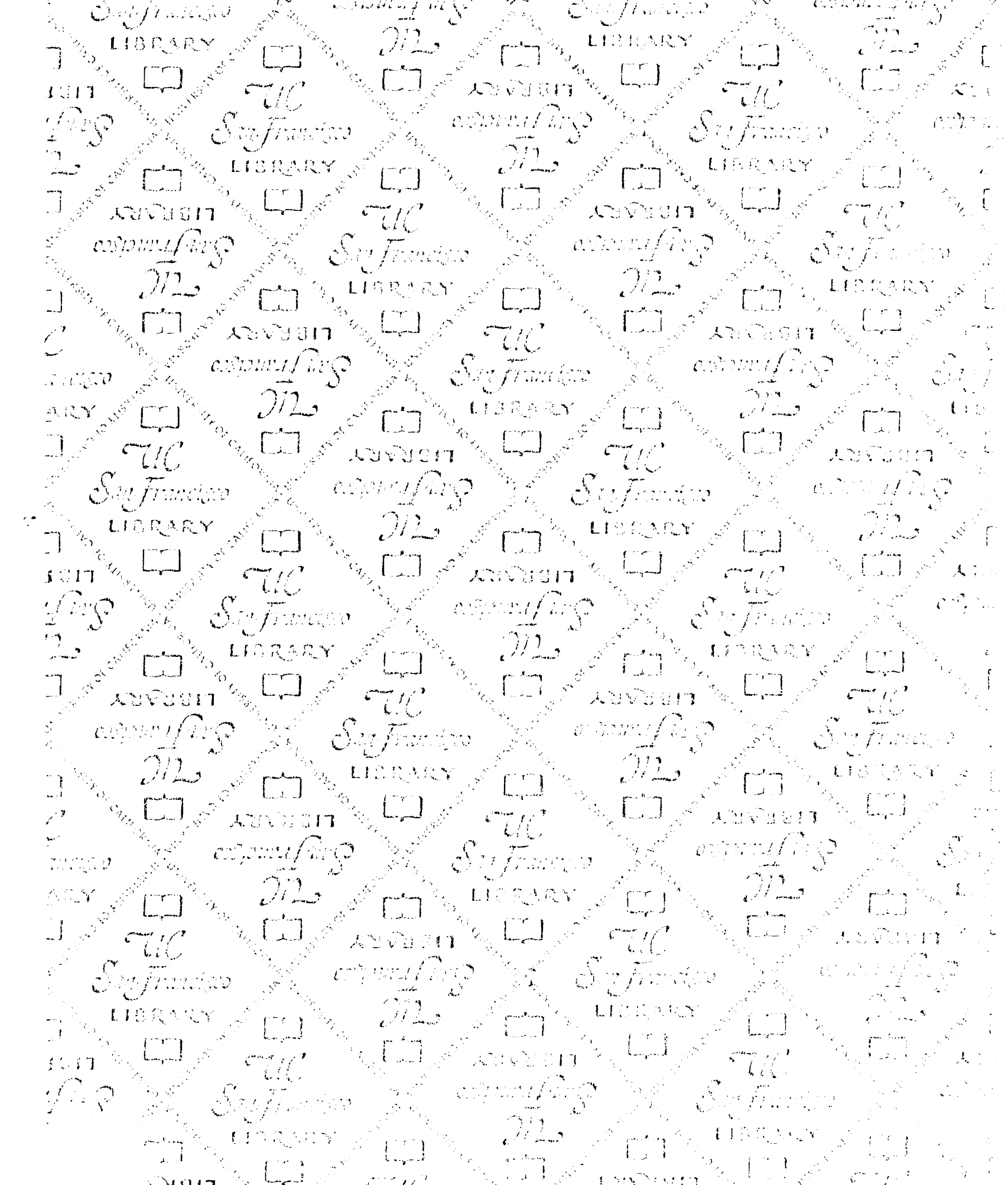


Figure 6 – Immunohistochemical analysis of α-syn expression in DA (TH+) neurons. These photos were taken from a single asyn-3 F1 heterozygote which was processed through IHC to label DA neurons red and α-syn green. While many more DA neurons express detectable levels of α-syn, such DA neurons were still a minority. In addition, these α-syn-expressing DA neurons are largely confined to the telencephalon and are not observed in more posterior portions of the brain.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