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An inducible model of human amylin overexpression reveals diverse transcriptional changes

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Abstract

Human Islet Amyloid Polypeptide or amylin is a neuroendocrine peptide with important endocrine and paracrine functions. Excessive production and accumulation of human amylin in the pancreas can lead to its aggregation and apoptosis of islet β -cells. Amylin has been shown to function within the central nervous system to decrease food intake, and more recently, it has been revealed that amylin is directly transcribed from neurons of the central nervous system, including the hypothalamus, arcuate nucleus, medial preoptic area, and nucleus accumbens. These findings alter the current model of how amylin targets the nervous system, and as a result may lead to obesity and type II diabetes mellitus. Here we set out to use *Caenorhabditis elegans* as an inducible *in vivo* model system to study the effects of amylin overexpression in tissues that include the nervous system. We profiled the transcriptional changes in transgenic animals expressing human amylin through RNA-seq. Using this genome-wide approach our results revealed for the first time that expression of human amylin in tissues including the nervous system induce diverse physiological responses in various signaling pathways. characterization of transgenic *C. elegans* animals expressing human amylin, we also observed specific defects in neural developmental programs as well as sensory behavior. Taken together, our data demonstrate the utility of using *C. elegans* as a valuable *in vivo* model to study human amylin toxicity.

Keywords

C. elegans; hyperamylinemia; neurons; RNA-seq; transcriptome

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Author Contributions

DMO and AJ conceived and supervised the study; DMO, YA, K.B., D.C.B., AJ, and SS designed experiments; YA, DMO, K.B., D.C.B., and SS performed experiments; DMO, YA, AJ, K.B., D.C.B., and SS analyzed data; DMO, AJ, K.D., D.C.B., YA, and SS wrote the manuscript.

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Conflict of interest

The authors declare that they do not have any conflict of interest.

1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disease that has been the focus of medical study for millennia [16]. The disease is manifested in two forms: type I diabetes mellitus, or juvenile diabetes, and type II diabetes mellitus, or adult-onset diabetes. It is estimated that in 2010, 285 million adults worldwide had diabetes [33]. Approximately 90% of diabetes cases worldwide are attributed to type II DM, which is associated predominantly with human behavior and lifestyle [42]. Type II DM is associated with a combination of genetic and environmental factors, like diet, adiposity and activity [14], with two underlying mechanisms: 1) desensitization of the body to insulin, or 2) decreased insulin secretion from pancreatic β -cells; both of which may occur and lead to hyperglycemia and hyperinsulinemia. Hyperinsulinemia caused by type II DM is correlated with a greater risk of developing neurological disorders, such as dementia and late-onset Alzheimer's disease (LOAD), which are two diseases with increasing prevalence among adults [23]. When associated with type II DM, dementia and LOAD are accompanied by defects in insulin-signaling within the CNS [10]. As the incidence of both diseases grows, research into the link between the pathology of type II DM and associated neurological disorders could offer insight into how to treat and mitigate the prevalence of both diseases.

One significant link in the pathology between type II DM and its associated neurological disorders is the pancreatic hormone amylin. Amylin, also known as islet amyloid polypeptide (IAPP), is a 37-amino acid peptide hormone that is co-secreted from the pancreatic islet β -cells with insulin [40]. Amylin has multiple physiological functions, including inhibition of glucose-stimulated insulin secretion, inhibition of glycogen synthesis in skeletal muscle and vasodilatation [27,39]. Amylin has also been demonstrated to have several effects on the CNS by regulating energy homeostasis during feeding and also inducing sympathetic nerve activity to peripheral tissues, like thermogenic brown fat [9,24]. Recently, it has been shown that amylin is transcribed widely in hypothalamic neurons of the central nervous system, where it has been shown to regulate feeding behavior through activation by leptin [20]. The link between amylin and the CNS has generated new questions on the relationship between type II DM and related neurological disorders. The role of amylin in type II DM has been characterized as the formation of amylin oligomers and amyloid fibrils, misfolded forms of the amylin protein, and the subsequent apoptotic effect of those amyloids on pancreatic β -cells that leads to insulin deficiency [29,34]. Amylin-derived amyloid deposits, or plaques, have been found in the pancreas of 90% of patients with type II DM and the amount of deposition correlates negatively with total β -cell mass [26,41]. Furthermore, there is evidence suggesting that human amylin plaques may play some role in neurodegeneration. Human amylin-derived protein aggregates have been recently found in plaques within the brain of patients with dementia, and Alzheimer's disease who also suffered from type II DM [12,35]. The amyloid-aspect of amylin pathogenesis offers a link to the pathology of other neurodegenerative disorders, such as LOAD. LOAD also exhibits an apoptotic amyloid plaque called amyloid β , or A β , and together, amylin and A β represent 2 of 29 proteins in the human body that feature this type of structural mis-folding into amyloids [4]. Amyloids feature in a variety of diseases (collectively referred to as amyloidosis) with similar pathology, and despite the lack of

homology between their native protein forms, the overlapping pathology implies they share common targets [8].

Recently, the function of amylin in the CNS *in vivo* has become the subject of study using both murine and fruit fly model organisms. One study has shown that overexpression of amylin can lead to amylin oligomerization and can cause neurological deficits in the brains of transgenic mouse models as well as a neuroinflammatory response [35]. In line with the neurotoxicity of human amylin, another study showed that increased levels of human amylin induce neuronal cell death via an amylin receptor dependent mechanism [13]. Toxic human amylin oligomers and aggregates have also been detected *in vivo* in transgenic *Drosophila melanogaster* and *Caenorhabditis elegans* models as well [30,31]. Rosas *et al* demonstrated that h-proIAPP expression in body-wall muscles, pharynx and neurons adversely affects *C. elegans* growth and development [30].

Here we build upon these efforts and present our data developing *Caenorhabditis elegans* as a model system for investigating inducible human amylin toxicity *in vivo*. We uncovered neural developmental and sensory behavior defects in transgenic animals expressing human amylin, and employed RNA-seq to transcriptionally profile changes in gene expression after induction of human amylin. Our results reveal that inducible expression of human amylin produces diverse physiological responses in various signaling pathways and taken together validates the utility of using *C. elegans* as a novel *in vivo* model to study adverse effects and mechanisms of human amylin toxicity.

2. Materials and Methods

2.1 Strains and maintenance

The *C. elegans* strains used in this project were N2 wildtype, CZ1200 *juIs76* [*prom_{unc-25}::GFP*; *lin-15(+)*] II; *lin-15B(n765)* X, DMH46 *hanEx25* [*prom_{hsp16.2}::humanAmylin*; *prom_{ofm-1}::GFP*], DMH46; *juIs76* [*prom_{unc-25}::GFP*; *lin-15(+)*] II. Animals were maintained on NGM plates seeded with *E.coli* strain OP50 and maintained at 20°C, unless otherwise noted, according to standard protocol [3].

2.2 Cloning human amylin

The cDNA encoding the mature (processed) 37-aa human amylin transgene was codon optimized for *C. elegans* expression and directly cloned into the plasmid pPD49.78 (AddGene) at *Bam*HI and *Kpn*II sites. The pPD49.78 plasmid contains the *hsp16.2* promoter, which drives expression in neurons, pharyngeal muscle, and hypodermal cells after heat induction. This transgene is designated *hanEx25*. The human amylin transgenic line was made via microinjection of the *hanEx25* transgene at 5ng/μl and co-injected with *prom_{ofm-1}::GFP* at 50 ng/μl into the germline cells of adult N2 animals. GFP positive offspring were cloned out and maintained to isolate the stable transgenic line named DMH46.

2.3 Western Blot Analysis and ELISA

To analyze the transgene expression, western blot was performed. Transgenic and wild type animals were collected and lysed in RIPA buffer followed by sonication in the presence of protease inhibitor cocktail on ice. Protein content in cell extract was determined using BCA protein quantification kit (Pierce). 30 µg of protein extract was added on 4–15% tris-glycine gels and resolved proteins were electro-transferred to nitrocellulose membranes. The blots were blocked in 5% milk for 1 hour, washed and probed with primary anti-human amylin mouse monoclonal or rabbit polyclonal antibodies (Santa Cruz) at a 1:250 dilution in PBS/Tween (0.1%). Following washing, the blot and then probed with the corresponding HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies at a 1:2000 dilution for 1hr. Blots were developed with a chemiluminescent ECL substrate (Pierce) and images were captured with the Kodak 1500 imaging station.

2.4 Transcriptional Analysis & RNA seq

The mRNA samples were sequenced using the HiSeq platform (Illumina) for 6 *C. elegans* samples (three replicates from two conditions) in order to generate 151 base pair reads. These paired end reads were populated into two separate FASTQ format files and the quality of the reads tested using the FastQC toolkit to ensure quality of the sequencing reads. The sequencing reads were used as input for the TopHat [15] read alignment tool to be aligned to the genomic *C. elegans* reference sequence (Ensembl version WBcel235.80) for each of the samples. TopHat aligns the sequencing reads to the reference genome using the reference index created by Bowtie [18,19], and then identifies the splice junctions from the reads aligning to multiple exons. The output from TopHat was obtained as BAM format files that consist of information on the alignment of each individual read within the reference genome and the splicing information of that read. In the alignment phase, we allowed up to two mismatches per 30 base pair segment and removed reads that aligned to more than 20 different genomic locations. The BAM alignment files obtained from the TopHat alignment tool were analyzed to generate the alignment statistics for each sample, namely, the total number of reads, the number of mapped reads and the percent of mapped reads. The alignment BAM files from TopHat were further utilized to compute gene expression levels and test each gene for differential expression. The number of reads that mapped to each gene described in the Ensembl annotation was calculated using the Python package HTSeq [2]. Differential gene expression analysis was conducted using the DESeq [1] R package (available from Bioconductor). The DESeq analysis resulted in the determination of potential differentially expressed genes when compared between the DMH46 and N2 (wildtype) samples. The read counts for each sample were normalized for sequencing depth and distortion caused by highly differentially expressed genes. Then the negative binomial (NB) model was used to test the significance of differential expression between two genotypes. The differentially expressed genes were deemed significant if the FDR (False Discovery Rate) was less than 0.05, and the gene expression was above the 10th percentile, and showed greater than 2-fold change difference (overexpressed or under expressed) between the conditions.

To validate differentially expressed genes, we selected four candidate genes based on significant fold differences across all samples and analyzed their relative mRNA levels using

qRT-PCR. Total RNA was extracted using Trizol Reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems). cDNA samples were diluted 1:10 in nuclease-free water and used as template for qRT-PCR experiments using the EXPRESS SYBR® GreenER kit with Premixed ROX (Invitrogen). All experiments were performed on a Mastercycler® ep realplex2 (Eppendorf) in 96-well plates following the manufacturer's instructions. For each sample, the amount of mRNA detected was normalized to mRNA values of the control housekeeping gene *rpl-32* (Ribosomal protein L32, WBGene00004446). Statistical analysis of qRT-PCR results represent the means and standard deviations of relative values from three biological replicates. Data were statistically analyzed using a one-way analysis of variance (ANOVA) with a Tukey post-hoc test for multiple comparisons.

2.5 Phenotypic analysis

Induction of human amylin (DMH46 line) was performed prior to chemoavoidance assays for 3 consecutive days (3 hours at 33°C). These heat inductions began on day 1 of adulthood, and therefore day 3 adults were assayed for behavior. Non-induced DMH46 animals as well as heat induced wildtype (N2) populations were also tested as controls. In each case animals were washed from plates using S-Basal and then washed two more times with S-Basal and once with ddH₂O. Chemoavoidance assays to the AWB sensed odor 2-nonanone was performed as described previously [37]. Assay plates were divided into 6 sections (A-F), and animals were placed on the center of the plate between sections C and D. Two spots of 1µl of 2-nonanone diluted in 10µl of 100% EtOH were placed at one end of the plate and two 1µl spots of 100% EtOH were aliquoted to the opposite end of the plate as a control. The chemotaxis index was calculated by taking the total number of animals that migrated to the A and B sections and subtracting the total number of animals that migrated to the E and F sections and then dividing this number by the total number of animals on the assay plate [11,37]. The assays were repeated on three separate days and between 50 and 200 animals were used for each assay.

DD and VD motor neuron commissural defects were quantified as described previously [22,32] by counting the number of wildtype and defective commissures from populations and calculating the percentage defect. Commissures were visualized by crossing DMH46 animals into the background of the CZ1200 strain which contains the transgene *juls76* [*prom^{unc-25}::GFP+lin-15(+)*] that expresses soluble GFP in the DD and VD GABAergic motor neurons. The number of normal or defective commissures from a population was counted and percent defects determined by summing all the defective commissures and dividing this by the total number of commissures. A defect was scored if the commissure could not be seen to connect with any part of the dorsal nerve cord. Penetrance was scored by calculating the percentage of animals across a population exhibiting at least one commissure connectivity defect. Three types of individual axon defects were distinguishable: wayward, premature branch and premature stop defects - the resulting defects were also binned into one of these categories. Between 50 and 100 animals were examined in each case. To induce human amylin expression prior to scoring the VD DD neurons for any potential defects, a timed-egg lay was performed by allowing gravid adults

to lay eggs on a seeded plate for 3hrs. All adults were removed from those plates and the remaining progeny subjected to heat-induction at 33°C for 3 hours. As well as DMH46 animals, control CZ1200 animals were subjected to the same protocol to serve as controls. After heat induction, animals were maintained under normal conditions for 3 days and scored at day 1 of adulthood.

3. Results

3.1 Inducible expression of human amylin in *C. elegans*

To express human amylin in the nervous system of *C. elegans* we cloned the human amylin gene under a heat inducible promoter (*hsp16.2*) that drives expression predominately in hypodermal cells and neurons (Figure 1A). Using this approach, we were able to generate a stable transgenic line of *C. elegans* animals expressing this transgene (DMH46). To ensure that expression of human amylin could be induced by heat, we subjected DMH46 animals to higher temperatures (33°C) for 3hrs and tested for the presence of human amylin protein by Western blot (Figure 1B). As expected, using a monoclonal antibody specific to human amylin, we could detect the presence of human amylin in DMH46 animals after heat-induction. Furthermore, we found that repeating the heat induction procedure on four consecutive days resulted in increasing levels of human amylin in DMH46 animals (Figure 1B). In wildtype control animals, no amylin protein was detected even after prolonged heat stress, due to the absence of a gene encoding amylin in the *C. elegans* genome. We also performed Enzyme-Linked Immunosorbent Assays (ELISA) to obtain quantitative measures of amylin expression after heat induction, and similarly observed no detectable amylin expression in wildtype animals or un-induced amylin containing animals (DMH46), and significant amylin protein levels by day three of induction as compared with controls (data not shown). From these data we demonstrate that human amylin can be expressed within *C. elegans* in a temporally and dose dependent manner after heat induction.

3.2 Expression of human amylin in *C. elegans* influences diverse cellular pathways

In order to understand how the molecular landscape changes in *C. elegans* after induction of human amylin, we performed RNA-seq analysis on DMH46 animals after induction of human amylin and on wildtype animals that were subjected to the same heat induction protocol. In order to ensure amylin was induced in the case of DMH46 transgenic animals, we performed chemoavoidance assays prior to RNA isolation (see section 3.3 below for details on chemoavoidance defect). From these assays, we could behaviorally confirm that human amylin was overexpressed using defects in chemoavoidance behavior as our readout ($p < 0.001$ for DMH46 after heat induction versus wildtype animals after heat induction). We examined the transcriptional profile in triplicate for each genotype (i.e. wildtype + heat, and DMH46 + heat). 82% of reads mapped to the *C. elegans* genome (Figure 2A) and reads mapping to the human amylin gene were only obtained for DMH46 and not for wildtype animals. The quantitative real-time RT-PCR (qRT-PCR) analysis of four randomly selected genes (*nca-2*, *unc-10*, *y38h6c.23*, *ugt-36*) using gene-specific primers validated the RNA-Seq data (Figure S1). Upregulation of *nca-2* and *unc-10* and downregulation of *y38h6c.23* and *ugt-36* was observed via RNA-seq and also qRT-PCR. Interestingly, we observed a significantly larger number of downregulated genes (280) compared with upregulated genes

(63). However, in each case diverse pathways and types of proteins are altered (Figure 2B–2C). The Protein ANalysis THrough Evolutionary Relationships (PANTHER [25,36]) database was probed to categorize gene IDs into biological processes and types of proteins. The largest category of upregulated genes belonged to metabolic pathways and when categorized by protein type the largest subset of upregulated genes were receptor type proteins, followed next by transcription factors and then nucleic acid binding proteins; we also observed significant increase in the expression of the GABAergic gene *gbb-1* which encodes a subunit of the GABA-B receptor (1.9 Log Fold Change in DMH46 versus wildtype). In the case of genes being turned down, the largest groups of downregulated genes was involved in metabolic processes that included oxidoreductases and transferases, followed next by structural proteins (Figure 2C). When the downregulated genes are organized by pathway, we observed a very diverse list of 44 pathway hits, the highest of which was related to the proteasome, which has been shown to be a critical structure in ameliorating the toxicity effect of aggregation prone amyloid proteins including human amylin [5,7,38]. Additionally, *fbxb* genes like *fbxb-42*, *fbxb-77*, *fbxb-81* and *fbxb-96* from the Fbox family, known to play a role in ubiquitin mediated proteolysis [17] were upregulated. We presented the top 20 upregulated and downregulated genes that were identified in both the control (wildtype) and the experimental (DMH46) but were only upregulated in DMH46 relative to wildtype (Figure 3A) or downregulated in DMH46 relative to wildtype (Figure 3B). The upregulated list is a mixture of transcriptional regulators, calcium channels, receptors, and transporters. The downregulated list is also diverse and includes metabolic pathway enzymes such as dehydrogenases (fatty acid dehydrogenase, *fat-7*), transferase, transporters, fatty acid dehydrogenase, *fat-7* as well as heat shock proteins *hsp-16* and *hsp-17*. These results are in line with a previous cellular study [21], which showed that human amylin and β -amyloid target and downregulate a common set of metabolic proteins such as dehydrogenases IDH3A, GAPDH, PHGDH, MDH1, LDHA and transferases GOT2 and SHMT2. There was also an interesting list of genes that were turned up in DMH46 but with no reads mapped for these genes in wildtype animals – *F07E5.12*, *Y60C6A.2*, *C33D3.6*, *fbxb-42*, *Y48G9A.7*, *fbxb-77*, *H24K24.2*, *F54G2.2*, *ZC84.7*, *Y47H10A.3*, *R07D5.2*, *klf-2*, *tab-1*, *fmo-2*, and *ceh-30*. In the case of these genes it was not possible to calculate a Log Fold Change (LFC) as they were only mapped from one of the genotypes. These genes included some uncharacterized genes, as well as numerous transcriptional regulators including multiple transcription factors that have been implicated in neuronal cell fate specification.

3.3 Expression of human amylin affects developmental programs as well as dynamic sensory processing in *C. elegans*

Next, we sought to understand the consequence of overexpressing human amylin in *C. elegans*. We investigated both developmental and dynamic processes within the nervous system of *C. elegans*. We firstly examined the avoidance response of *C. elegans* animals expressing human amylin in the nervous system. *C. elegans* uses a pair of head neurons termed the AWB, to detect volatile repellents like 2-nonanone. When presented with a point source of 2-nonanone, *C. elegans* will chemotax away from the point source of this odor (see Figure 4A for assay setup). Starting on day one of adulthood, we induced expression of human amylin in *C. elegans* transgenic animals for three consecutive days (Figure 4A(i)).

On day three of adulthood, we tested the ability of these animals to avoid the repellent odor, 2-nonanone. We also subjected wildtype N2 animals to the same protocol to control for the effect of heat on chemoavoidance. From these data we found that animals expressing human amylin were defective in their ability to avoid the repellent odor, 2-nonanone ($p < 0.005$ for DMH46 versus wildtype animals subjected to the same protocol – Figure 4B). We also did not observe any difference in the response of wildtype animals that were subjected to the same heat-induction protocol versus wildtype animals that were cultivated at consistent temperature ($p > 0.05$ – Figure 4B). As a control, we also examined speed of locomotion after the heat induction protocol depicted in Figure 4A(i) and observed no significant difference between amylin expressing DMH46 and wildtype animals (DMH46 average: $123\mu\text{m sec}^{-1}$ and N2 average: $142\mu\text{m sec}^{-1}$, $p > 0.05$). Examination of locomotion speed was performed as described previously [28].

Next, we tested the effects of overexpressing human amylin in *C. elegans* on neuronal development. To this end, we used the GABAergic ventral cord commissural neurons as our readout for functionality. This circuit is composed of two classes of neurons – the VD and the DD type neurons. The DD type neurons are born during embryogenesis (DD1 at 5hrs post fertilization), whereas the VD type neurons are born at the end of the first larval stage. *In utero* development in *C. elegans* last for ~2hrs, therefore, to examine the effect of overexpressing human amylin on the development of this circuit we tried to induce expression of human amylin prior to (in the case of the VD), or during (in the case of the DD), the birth of these neurons. We set up a timed egg lay and subjected these eggs to a heat induction cycle for 3hrs at 33°C (see Figure 4A(ii)). We then allowed the eggs to develop to day 1 of adulthood under normal cultivation temperatures and scored the number of commissures that failed to reach the dorsal nerve cord (see example of circuit in Figure 4C). As controls we included wildtype animals that were cultivated at normal temperatures and also subjected to the heat induction procedure during embryonic development. From these experiments, we found that transgenic animals expressing human amylin exhibited significant defects in the number of commissures that failed to reach the dorsal nerve cord in comparison to wildtype animals ($p < 0.005$ – Figure 4D). We did not observe any difference in wildtype animals that were subjected to the same embryonic heat induction versus wildtype animals that were consistently cultivated at standard temperature ($p > 0.05$ – Figure 4D). The penetrance was also compared between animals containing the human amylin transgene (DMH46 +) to animals that do not harbor the human amylin transgene (DMH46 -), and here we observed a change from 32% in DMH46(-) animals to 76% in DMH46(+) animals (Figure 4E). We also observed that the types of commissural guidance defects could be categorized into three main groups: 1) wayward connections; 2) premature branch; 3) premature stop. We analyzed our data on commissural guidance in wildtype animals that were subjected to embryonic heat induction and compared these data to DMH46 animals that also underwent embryonic heat induction and found that DMH46 animals that underwent amylin induction exhibited a greater proportion of premature branching defects, and very few premature stop defects. In the case of wildtype animals, we observed a similar number of defects in each category (Figure 4F). Taken together, these data suggest that overexpression of the aggregation prone, mature form of human amylin results in defects that were not observed in wildtype animals. Furthermore, the defects we observed in

DMH46 animals expressing human amylin were more likely to result in premature branching of migrating commissures rather than wayward or premature stop defects, suggesting that human amylin may interfere with regulators of axon guidance and induce branching prematurely before the growth cone reaches the dorsal nerve cord by affecting the interpretation of guidance cues.

4. Discussion

Using *C. elegans* we have been able to effectively overexpress human amylin with temporal control. From our experiments we observed defects in both sensory processing as well as developmental programs required to build neural circuits in relation to overexpression of the mature (processed) human amylin form. By using olfactory aversion as a read-out of sensory function, we uncovered defects related to human amylin overexpression that was not observed in wildtype animals. The specificity of defects related to human amylin overexpression was also observed by examining early developmental programs that influence neural circuit assembly. Together, this suggests that excessive accumulation of human amylin likely represents a necessary step in triggering specific defects in nervous system development and function. To develop a detailed understanding of the adverse effects of human amylin accumulation in CNS, we examined how the molecular landscape changes as a result of amylin overexpression. To achieve this goal, we designed RNA-seq experiments comparing the transcriptional profile of transgenic *C. elegans* animals overexpressing human amylin as compared to wildtype animals. Using this approach, we have uncovered a diverse set of targets for human amylin that included a large subset of metabolic pathway regulators whose expression is altered by the burden of amylin overexpression. Of particular note were genes involved in proteasome function, as a reduction in proteasome activity was shown previously in cultured human pancreatic β cells that were treated with human amylin [5] and islets procured from type-2 diabetics [6], and thereby suggesting that in both *C. elegans* and β cells, the proteasome may represent an important substrate for amylin during disease and vice versa.

Our results build on previous data [30] developing *C. elegans* as a model to study human amylin accumulation. The utility of our work is that we developed a system to control amylin induction with temporal control which we then leveraged to describe genome-wide transcriptional changes induced by human amylin overexpression by employing RNA-seq analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- A novel inducible *in vivo* system of human amylin overexpression
- Amylin overexpression results in sensory and neural development defects
- Expression of human amylin induces diverse physiological responses in various signaling pathways

Figure 1. Plasmid construction and expression of human amylin transgene in *C. elegans*. (A) The cDNA encoding the biologically active (mature) form of human amylin for *C. elegans* expression was cloned into the plasmid pPD49.78 (AddGene) at *Bam*HI and *Kpn*I sites. The pPD49.78 plasmid contains the *hsp16.2* promoter, which drives expression in neurons and hypodermal cells after heat induction; this transgene was introduced into wildtype animals via germ-line injection to generate the strain DMH46. (B) The induction of human amylin in strain DMH46 expression was performed by transferring a population of animals to 33°C for 3 hours. To detect the amylin protein, a monoclonal antibody specific to human amylin was used and revealed increased expression correlating with increased heat induction. In each case the actin protein was used as a loading control. wt indicates the wildtype control (N2) strain.

Figure 2. Human amylin overexpression in *C. elegans* affects diverse cellular pathways. (A) Transcriptome summary from DMH46 animals expressing human amylin versus wildtype animals that were subjected to the same induction protocol. The table displays the total number of reads as well as the number and percentage of reads mapped to the *C. elegans* genome, and also the percent mapped to exonic, intronic, or intergenic regions. (B) The PANTHER [25,36] database was used to categorize gene IDs into biological processes and types of proteins.

Figure 3. Human amylin overexpression induces the expression of diverse subsets of genes in *C. elegans*.

(A) The 20 most strongly upregulated and (B) downregulated genes upon induction of human amylin compared to wildtype control animals. X-axis represents the relative Log-Fold Change (LFC) for each gene after normalization against control animals. All genes have a fold-change higher than ± 2 . The upregulated list is a mixture of transcriptional regulators, calcium channels, receptors, and transporters. The downregulated list is also diverse and includes, dehydrogenases, transferase, transporters, and also the fatty acid dehydrogenase, *fat-7*.

Figure 4. Human amylin-induced behavioral and developmental defects in *C. elegans*.

Upper panels indicate the heat induction schemes used: (i) heat-induced amylin expression was invoked on three subsequent days from Day 1 of adulthood to Day 3 for 3hrs on each day, and in (ii) heat-induced amylin expression was invoked only once at the egg stage for 3hrs. The induction protocol in (i) was used to examine chemoavoidance behavior on Day 3 adults, and the protocol in (ii) was used to examine development of GABAergic neurons. For assaying development, animals were scored on Day 1 of adulthood. L1-L4 indicate larval stages 1 through 4. Lower panels indicate the chemoavoidance scheme for the AWB sensed odor 2-nonanone. *ct* indicates control odor, and *ori* indicates the animals starting point. **(B)** Chemotaxis response in wildtype and human amylin expressing transgenic animals (DMH46) prior to and following heat induction. DMH46 animals expressing human amylin were defective in their ability to avoid the repellent odor 2-nonanone after heat induction ($p < 0.005$ for DMH46 versus wildtype animals subjected to the same protocol). **(C)** Representative image of the GABAergic motor neuron circuit in a wildtype worm (CZ1200).

The GABAergic circuit is composed of DD and VD type motor neurons that were used to quantify commissural defects. **(D)** Defects in commissural guidance were visualized in DMH46 animals after heat induction (last bar) as compared to controls. **(E)** The penetrance of the commissural guidance defects is shown for animals containing the human amylin transgene (DMH46 +) and animals that do not harbor the human amylin transgene (DMH46 -). **(F)** Effect of hyperamylinemia on three types of commissural guidance defects observed: 1) wayward connections; 2) premature branch; 3) premature stop. Error bars represent the S.E.M. * indicates a $p < 0.05$ for the differences between transgenic animals and wildtype animals as well as transgenic animals subjected to heat versus transgenic animals not subjected to heat exposure. p values were calculated using the Student's t-test (Graphpad Prism 7).