New Alzheimer Amyloid β Responsive Genes Identified in Human Neuroblastoma Cells by Hierarchical Clustering

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Abstract

Alzheimer’s disease (AD) is characterized by neuronal degeneration and cell loss. Aβ42, in contrast to Aβ40, is thought to be the pathogenic form triggering the pathological cascade in AD. In order to unravel overall gene regulation we monitored the transcriptomic responses to increased or decreased Aβ40 and Aβ42 levels, generated and derived from its precursor C99 (C-terminal fragment of APP comprising 99 amino acids) in human neuroblastoma cells. We identified fourteen differentially expressed transcripts by hierarchical clustering and discussed their involvement in AD. These fourteen transcripts were grouped into two main clusters each showing distinct differential expression patterns depending on Aβ40 and Aβ42 levels. Among these transcripts we discovered an unexpected inverse and strong differential expression of neurogenin 2 (NEUROG2) and KIAA0125 in all examined cell clones. C99-overexpression had a similar effect on NEUROG2 and KIAA0125 expression as a decreased Aβ42/Aβ40 ratio. Importantly however, an increased Aβ42/Aβ40 ratio, which is typical of AD, had an inverse expression pattern of NEUROG2 and KIAA0125: An increased Aβ42/Aβ40 ratio up-regulated NEUROG2, but down-regulated KIAA0125, whereas the opposite regulation pattern was observed for a decreased Aβ42/Aβ40 ratio. We discuss the possibilities that the so far uncharacterized KIAA0125 might be a counter player of NEUROG2 and that KIAA0125 could be involved in neurogenesis, due to the involvement of NEUROG2 in developmental neural processes.

Introduction

Amyloid beta precursor protein (APP), presenilin 1 (PS1), presenilin 2 (PS2) and apolipoprotein E (APOE) have been associated with AD [1] and further susceptibility genes are expected to exist. The amyloid cascade hypothesis suggests that Aβ1–42 accumulation is the primary event in the pathogenesis of AD. Aβ1–42, two amino acids (isoleucine and alanine) longer than Aβ1–40, is more prone to aggregate and expected to trigger the pathological cascade in AD [2,3]. APP is cleaved by β-secretase resulting in the generation of the C-terminal fragment C99, which is further cleaved by the γ-secretase complex generating different Aβ species. By overexpressing C99 wildtype and C99 mutations, known to generate different Aβ42/Aβ40 levels [4,5] in human neuroblastoma cells, we obtained information about the genome-wide gene regulation by using whole genome microarrays.

We unexpectedly identified NEUROG2 and KIAA0125 as inversely regulated by an altered Aβ42/Aβ40 ratio. Remarkably, an increased Aβ42/Aβ40 ratio that is typical of AD inverted the expression pattern of NEUROG2 and KIAA0125 that was observed for a decreased ratio. Importantly, for a decreased Aβ42/Aβ40 ratio NEUROG2 and KIAA0125 were the most differentially expressed transcripts out of approximately 40,000 tested.

Neurogenesis in AD is a controversial topic. In hippocampi of patients with AD [6], where it may produce cells to replace neurons lost in the disease [7], neurogenesis has been reported to be enhanced [8]. This could be reproduced in a transgenic mouse model [9] in which APP mutations led to increased incorporation of bromodeoxyuridine (BrdU) and enhanced expression of immature neuronal markers in two neuroproliferative regions: The subventricular zone and the dentate gyrus. In contrast to this, neurogenesis has been reported to be decreased in mouse models for AD [10,11]. NEUROG2 plays an essential role in the development of the dentate gyrus of the hippocampus [12], which is the central structure for learning and memory processes. The impairment of neurogenesis in a mouse model exhibiting progressive amyloid deposition was reflected by a reduction in the number of neural stem cells, progenitor cells and neuroblasts in the dentate gyrus [13]. Our work provides a possible mechanism of how neurogenesis might be influenced, namely by the previously undiscovered inverse regulation of NEUROG2 and KIAA0125 caused by Aβ.

Results

We investigated the transcriptomic response to a changed Aβ42/Aβ40 ratio in human neuroblastoma cells. Constructs encoding the C-terminal part of APP (C99) were used to transfect human neuroblastoma cells in order to overexpress C99 [4,5].
Subsequently, overexpressed C99 was processed by γ-secretase releasing several proportions of Aβ12 and Aβ40. To modify the Aβ12/Aβ40 ratio we made use of two well established constructs [4,5]. The single point mutation I45F in C99 results in a strong overproduction of Aβ12 and a concomitant loss in Aβ40. The C99 point mutation V50F results in almost exclusive Aβ40 production. Total Aβ production from these constructs was similar in all clones and the clones used were expression level matched.

**SH-SYSY cells overproduced different Aβ42 and Aβ40 levels**

The human neuroblastoma cell line SH-SYSY was stably transfected with constructs coding for the APP C-terminal fragment C99WT, moreover with constructs bearing the point mutations C99I45F and C99V50F and the vector control (mock-transfected, negative control). The point mutations were utilized to shift the Aβ12/Aβ40 ratio in either direction. As was described in detail [4,5] C99V50F and C99I45F had an opposite effect on the Aβ peptides generated: As compared to the C99WT-transfected cells, the C99V50F-transfected cells expressed more Aβ40 and less Aβ42, whereas the C99I45F-transfected cells expressed more Aβ12 and less Aβ40 (Fig. 1).

Single independent cell clones (C99WT, C99V50F, C99I45F, mock-transfected negative control, n = 3 per group) were selected and used for whole genome microarray analysis. HG-U133 A and B microarrays (Affymetrix) were used (unprocessed and processed microarray data can be accessed via the ArrayExpress database, accession number E-MEXP-1913). Replicates from different independent clones were prepared and hybridized to the microarrays on different days. This procedure increased data variation between replicates, but more importantly, is expected to have increased accuracy (Fig. 2).

Hierarchical clustering of Alzheimer Amyloid β Responsive Genes

In order to investigate the overall gene regulation and to discover novel expression patterns, all tested cell clones were used for cluster analysis. Hierarchical clustering is used to group genes according to common properties, for instance their expression levels. With this information one can gain insight into relationships between genes or their proteins. Certain expression patterns represent common regulatory processes, which can be a sign of putative functional relationships. To obtain insight into the gene regulation in all cell clones, gene expression values of three independent replicates of C99WT, C99I45F, C99V50F and mock-transfected cells were clustered (Fig. 3).

The cluster analysis revealed a previously unknown inverse regulation of NEUROG2 and KIAA0125 in consequence of an inverse Aβ12/Aβ40 ratio. Interestingly, both genes were the most differentially expressed ones and importantly, they were inversely regulated in all measured cell clones.

To focus specifically on Aβ effects, it was essential to compare suitable cell clones. While comparisons of C99WT, C99I45F and C99V50F with mock-transfected cells provided information about C99-overexpression effects, comparisons of C99I45F and C99V50F with C99WT provided information about Aβ effects (Table 1). Our goal was to obtain information about Aβ12/Aβ40 effects, therefore we first concentrated on C99V50F, generating a decreased Aβ12/Aβ40 ratio, and compared it to C99WT generating medium Aβ12/Aβ40 levels. Whole genome expression profiling of these cell clones resulted in a gene list in which the genes were sorted according to their differential expression levels (Table 2). The first most up-regulated and first most down-regulated genes were then further investigated: We examined how...
these two genes were expressed when the Aβ_{42}/Aβ_{40} ratio was increased. Since an increased Aβ_{42}/Aβ_{40} ratio, as this is the case with C99I45F-transfected cells, is characteristic of AD, this approach was expected to provide essential information about the pathomechanism of AD.

Neurogenin 2 was strongly down-regulated, whereas KIAA0125 was strongly up-regulated in consequence of a decreased Aβ_{42}/Aβ_{40} ratio

The cells expressing a decreased Aβ_{42}/Aβ_{40} ratio (C99V50F) were compared to the cells transfected with the wildtype construct (C99WT). C99V50F expresses a smaller Aβ_{42}/Aβ_{40} ratio compared to the Aβ_{42}/Aβ_{40} ratio in C99WT (Fig. 1). Fourteen significantly dysregulated transcripts (seven up-regulated and seven down-regulated ones) were identified with NEUROG2 and KIAA0125 being the most prominent (Fig. 4). The corresponding probe set identification numbers (Probe set IDs), p-values, fold changes, gene symbols, gene titles and chromosomal locations are listed in Table 2.

Real-time PCR validated the inverse expression of NEUROG2 and KIAA0125 and revealed a correlation to relative Aβ_{42} levels

NEUROG2 and KIAA0125 transcription levels were measured by quantitative real-time PCR and compared to relative Aβ_{42} levels (Fig. 5A, 5B).

NEUROG2 gene expression correlates positively with relative Aβ_{42} levels as demonstrated by real-time PCR

Relative Aβ_{42} levels were ranked from high to low and plotted against the differential NEUROG2 expression (Fig. 5A). Here, we demonstrated that NEUROG2 expression increased together with increasing relative Aβ_{42} levels. NEUROG2 showed the strongest up-regulation (9.0 fold, S.E.M. = 2.3) for mutant C99I45F versus C99V50F, for which high relative Aβ_{42} levels were generated. NEUROG2 showed up-regulation (2.3 fold, S.E.M. = 0.6) for the comparison C99I45F versus C99WT; for this comparison medium relative Aβ_{42} levels were generated. NEUROG2 was down-regulated 4.0 fold (S.E.M. = 3.7) in mutant C99V50F compared...
KIAA0125 gene expression correlates negatively with relative Aβ$_{42}$ levels as demonstrated by real-time PCR

Relative Aβ$_{42}$ levels were ranked from high to low and plotted against the differential KIAA0125 expression (Fig. 5B). KIAA0125 showed an inverse expression pattern to that of NEUROG2 in response to increased relative Aβ levels. KIAA0125 showed the strongest down-regulation (9.8 fold, S.E.M. = 2.6) for the comparison of mutant C99I45F versus C99V50F, for which the highest relative Aβ$_{42}$ levels were generated. KIAA0125 was down-regulated 1.8 fold (S.E.M. = 0.5) for the comparison C99I45F versus C99WT (medium relative Aβ$_{42}$ levels) and it was up-regulated 5.4 fold (S.E.M. = 0.9) in mutant C99V50F compared to C99WT, for which the lowest relative Aβ$_{42}$ levels were generated.

NEUROG2 and KIAA0125 are inversely regulated in all tested cell clones

Comparing the gene expression of NEUROG2 and KIAA0125, measured by real-time PCR, confirmed the results revealed by microarray analysis: The stronger the NEUROG2 up-regulation in certain cell clones (Fig. 5A), the stronger is the KIAA0125 down-regulation in the same cell clones (Fig. 5B) and vice versa. C99-overexpression had a similar effect on NEUROG2 and KIAA0125 expression as a decreased Aβ$_{42}$/Aβ$_{40}$ ratio. Importantly however, an increased Aβ$_{42}$/Aβ$_{40}$ ratio, which is typical of AD, had an inverse expression pattern of NEUROG2 and KIAA0125. A

Figure 3. Hierarchical clustering of transcripts according to their expression values. Gene expression values of three independent replicates of C99WT, C99I45F, C99V50F and mock-transfected cells were clustered in order to obtain information about expression patterns. Two main clusters were identified. An inverse Aβ$_{42}$/Aβ$_{40}$ ratio resulted in an inverse NEUROG2/KIAA0125 regulation. Genes were clustered with the Manhattan metric as distance between the centered expression profiles and complete linkage as distance between the clusters. GC-RMA normalized m-RNA levels were used. This procedure revealed a so far unknown correlation between NEUROG2 and the previously uncharacterized KIAA0125: NEUROG2 and KIAA0125 were inversely regulated. Here, no baseline experiment was defined. Instead, for each probe set the mean over all chips was calculated and was subtracted from every single value (centering of data). The fold change refers to the mean of all expression values: NEUROG2 (marked as 1A in Fig. 3) is up-regulated approximately 8 fold (dark red colour) compared to the average expression of all chips appearing in Fig. 3. The average expression of all chips is regarded as a suitable baseline in order to see overall gene regulation in all cell clones. The yellow frames indicate the inverse regulation of NEUROG2 and KIAA0125 in the cells expressing inverse Aβ$_{42}$/Aβ$_{40}$ ratios: C99I45F (Aβ$_{42}$/Aβ$_{40}$↑) up-regulated NEUROG2, but down-regulated KIAA0125, whereas C99V50F (Aβ$_{42}$/Aβ$_{40}$↓) down-regulated NEUROG2, but up-regulated KIAA0125 in all triplicates. Triplicates were derived from independent single clones so that clonal effects were highly unlikely. Interestingly, the replicate in which the strongest up-regulation was observed (1A) also showed the strongest down-regulation for KIAA0125 (1B) and vice versa (2A and 2B). doi:10.1371/journal.pone.0006779.g003
Table 1. Comparisons between cell clones and information obtained about C99 and Aβ-effects.

| Comparisons | Effects |
|-------------|---------|
| C99WT/mock  | C99 overexpression |
| C99145F/mock| C99 overexpression+Ab42 overproduction |
| C99V50F/mock| C99 overexpression+Ab40 overproduction |
| C99145F/C99WT| Ab42 overproduction |
| C99V50F/C99WT| Ab40 overproduction |

doi:10.1371/journal.pone.0006779.t001

Table 2. Most up and down-regulated transcripts derived from the comparison C99V50F/C99WT1 (Ab42/Ab40 ↓).

| Position | Probe set ID | p-value | Fold change C99V50F/C99WT1 | Gene symbol | Gene title | Chromosomal location |
|----------|--------------|---------|----------------------------|-------------|------------|----------------------|
| 1        | 206478_at    | 0.00142 | 5.3                        | KIAA0125    | mitogen-activated protein kinase 8 interacting protein 2 | chr14q32.33 |
| 2        | 206803_s_at  | 0.00191 | 2.5                        | MAPKBP2     | actin, alpha 2, smooth muscle, aorta | chr20q13.33 |
| 3        | 200974_at    | 0.00115 | 2.2                        | ACTA2       | actin, alpha 2, smooth muscle, aorta | chr10q23.3 |
| 4        | 216963_s_at  | 0.00449 | 2.2                        | GAP43       | growth associated protein 43 | chr3q13.1-q13.2 |
| 5        | 201860_s_at  | 0.00477 | 2.1                        | PLAT        | plasminogen activator, tissue | chr8p12 |
| 6        | 206397_x_at  | 0.00269 | 2.1                        | GDF1        | growth differentiation factor 1 | chr18p12 |
| 7        | 204471_at    | 0.00190 | 2.1                        | GAP43       | growth associated protein 43 | chr3q13.1-q13.2 |
| 7        | 220287_at    | 0.00110 | −2.0                       | ADAMTS9     | a disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 9 | chr3p14.3-p14.2 |
| 6        | 209220_at    | 0.00089 | −2.5                       | GPC3        | glypican 3 | chr17q25.1 |
| 5        | 212148_at    | 0.00288 | −2.8                       | PBK1        | pre-B-cell leukemia transcription factor 1 | chr1q23 |
| 4        | 209757_s_at  | 0.00416 | −3.0                       | MCVN        | v-myc myeloblastosis viral related oncogene, neuroblastoma derived (avian) | chr2p24.1 |
| 3        | 202283_at    | 0.00362 | −3.1                       | SERPINF1    | serine protease inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1 | chr17p13.1 |
| 2        | 209238_at    | 0.00268 | −3.7                       | STX3A       | syntaxin 3A | chr11q12.1 |
| 1        | 215632_at    | 0.00166 | −12.5                      | NEUROG2     | neurogenin 2 | chr4q25 |

Table 2 Most up and down-regulated transcripts derived from the comparison C99V50F/C99WT1 (Ab42/Ab40 ↓). n = 3 per group; p < 0.005, unadjusted p-values; C99WT1 was the baseline experiment. The unspecific GAP43 and its spliced isoform were detected by two different probe sets (204471_at and 216963_s_at).

doi:10.1371/journal.pone.0006779.t002
human protein reference database) or 7.7 kDa (according to the Uniprot database, or 7.7 kDa (according to the UniProt/Swiss-Prot databases). Provided the mRNA is actually expressed, the complete coding sequence is expected to be a peptide with 76 amino acids (according to the Uniprot database, based on the nucleotide sequence).

NEUROG2 is a member of the neurogenin subfamily of basic helix-loop-helix (bHLH) transcription factors that play an important role in neurogenesis. During mouse neurogenesis, NEUROG2 and NEUROG1 are expressed in distinct progenitor populations in the central and peripheral nervous systems [15]. Yan et al. observed that in the developing chick retina, NEUROG2 was expressed in a subpopulation of proliferating progenitor cells [16]. Scardigli et al. hypothesized that NEUROG2 is both responsive to, and a regulator, of genetic pathways that specify neuronal fates in the ventral spinal cord [17]. It has been shown that the development of mesencephalic dopamine neurons is severely compromised in NEUROG2-null mutant mice. NEUROG2 is required for the differentiation of ventricular zone progenitors into postmitotic dopaminergic neuron precursors in the intermediate zone. It was concluded that NEUROG2 is required for the development of midbrain dopaminergic neurons [18]. NEUROG2 was immunohistochemically detected in a certain cycling population during G1 phase and was further restricted during G2-M phases to the subventricular zone-directed population. NEUROG2 may further be involved in the asymmetric cell divisions of progenitor cells [19]. Moreover, it has been reported that inhibition of pronuclear bHLH factors, like NEUROG2, in cortical progenitors promotes the formation of astrocytes [20].

A changed $\frac{A\beta_{42}}{A\beta_{40}}$ ratio is typically found in familial Alzheimer’s disease (FAD) and we used the same terminology here. It should be noted however, that the basic increases or decreases of the individual $A\beta$ species levels might have biological effects, which can be independent of the $A\beta$ ratio [21,22]. Indeed, based on absolute $A\beta$ species levels (Supplementary Information, Table S2), the unbiased cluster analysis (Fig. 5) would be in agreement with an $A\beta_{42}$ effect. This is reflected by the grouping of mock and C99V50F in one main cluster and C99WT and C99I45F in another one, which is in agreement with the extent of absolute $A\beta_{42}$ levels.

We propose the following property/function to KIAA0125: Firstly, it can be speculated that KIAA0125 may act as an antagonist of NEUROG2, secondly, that inverting the $A\beta_{42}/A\beta_{40}$ ratio also inverts the expression of NEUROG2/KIAA0125, thirdly, it can further be speculated that KIAA0125 might play a role in neurogenesis, maybe in preventing the generation of dopaminergic neurons or it could also be involved in inducing astrocytosis.

It had not escaped our notice that also other genes are clustered in similar ways like KIAA0125 (for instance GAP43, see Fig. 3). However, these genes do not reach such extreme differential expression values like KIAA0125. Though, for such genes an important relationship to NEUROG2 may also exist. Moreover, it has to be taken into consideration that further genes exist, which do not pass our cut-off criteria for significance ($p < 0.005$); for some of these genes a relationship similar to that of KIAA0125 cannot be excluded.

Our work has not only been restricted to NEUROG2 and KIAA0125, but provides further information about the regulation of several transcripts involved in Aβ induced gene expression. While differential expression for NEUROG2 and KIAA0125 was validated by real-time PCR, any remaining genes listed in Table 2 were not validated by another method. However, using a rather stringent cut-off for significance ($p < 0.005$; unadjusted p-values) provided more confidence in these data than using the frequently used threshold of $p < 0.05$.

Interestingly, the KIAA0125 regulation was found to be similar to the one of growth associated protein 43 (GAP43) and plasminogen activator, tissue (PLAT). GAP43 has been termed a ‘growth’ or ‘plasticity’ protein because it is expressed at high levels in neuronal growth cones during development and axonal regeneration. It is considered to be a crucial component of an effective regenerative response in the nervous system. Phosphorylation of this protein by protein kinase C is specifically correlated with certain forms of synaptic plasticity. The fact that GAP43 was up-regulated in consequence of a decreased $A\beta_{42}/A\beta_{40}$ ratio, but not in response to an increased one, is in line with the aberrant GAP43 gene expression that has been observed in AD [23]. Furthermore, it has been demonstrated that treatment of neuronal cultures with $A\beta_{40}$ for four days dose-dependently increased GAP43 levels and it has been suggested that $A\beta_{40}$ may promote neurite formation in primary neuronal cultures [24].

Plasminogen activator, tissue (PLAT) is regarded as one of the top candidate genes in AD according to the Alzforum database (www.alzforum.org). It is one of the most prominent activators of fibrinolysis. Its up-regulation in consequence of a decreased $A\beta_{42}/A\beta_{40}$ ratio (wheras an increased ratio did not up-regulate PLAT) may contribute to enhanced fibrinolysis. This may offer an explanation for the increased tendency of having strokes in AD patients because for an increased $A\beta_{42}/A\beta_{40}$ ratio, typical of AD, this putative protective up-regulation of PLAT might be missing. Most interestingly, PLAT was found to be down-regulated 3.5 fold ($p = 0.0037$) in the brains of Down’s syndrome patients (see supplemental information published in [25]), who also have a greater prevalence of strokes [26] and in which $A\beta_{42}$ levels are increased due to a gene dosage effect caused by triplication of the APP gene localized on chromosome 21 (trisomy 21). These observations argue for a negative correlation between PLAT expression and the $A\beta_{42}/A\beta_{40}$ ratio in vitro and in vivo.

Fibrin, the end product of blood coagulation, can be converted into soluble fragments (fibrinolysis). Plasmin, a protease, converts
Figure 5. Relative gene expression of Neurogenin 2 and KIAA0125 measured by real-time PCR and compared to relative Aβ42 levels. Fig. 5A and 5B show an almost linear relationship of Neurogenin 2/KIAA0125 expression and relative Aβ42 levels. It is noteworthy that these relationships are in opposite directions: While Neurogenin 2 expression increases with increasing relative Aβ42 levels, KIAA0125 expression decreases with increasing relative Aβ42 levels. Importantly, the same regulation pattern was confirmed by real-time PCR as previously observed by microarray analysis: The stronger the Neurogenin 2 up-regulation in certain cell clones (Fig. 5A), the stronger was the KIAA0125 down-regulation in the same cell clones (Fig. 5B) and vice versa. Total-RNA was originated from the same clones as the ones used for the microarrays. This total-RNA was converted into cDNA and used for real-time PCR. Cyclophilin A expression was used for normalisation. Error bars represent the standard error of the mean (S.E.M.) of three independent cell clones.

doi:10.1371/journal.pone.0006779.g005
fibrin into soluble fragments by cleavage. The serine protease inhibitor, clade F, member 1 (SerpinF1, α-2 anti-plasmin) is an anti-plasmin and it was down-regulated 3.1 fold (p = 0.00362) for a decreased Aβ12/Aβ40 ratio. It can be speculated that more plasmin may be available, which in turn could accelerate fibrinolysis (for a decreased Aβ12/Aβ40 ratio).

Actin, alpha 2, smooth muscle, aorta (ACTA2) is one of six different actin isoforms. Actins are highly conserved proteins that are involved in cell motility and cell structure. They are ubiquitous proteins involved in the formation of filaments that are major components of the cytoskeleton. Interaction with myosin provides the basis of muscular contraction and many aspects of cell motility. ACTA2 was the third most strongly up-regulated gene for Aβ12/Aβ40 ratio, but it was not differentially expressed for Aβ12/Aβ40 ratio. It was co-regulated with GAP43, PLAT, GDF1 and MAPK3IP2. ACTA2 has been described as being involved in vascularisation and vascular branching [27,28]. Furthermore, impaired vascular contractility and blood pressure homeostasis in smooth muscle α-actin null mice have been observed [29]. The major function of vascular smooth muscle cells is contraction to regulate blood pressure and blood flow [30]. It can be speculated that upregulation of ACTA2 in consequence of a decreased Aβ12/Aβ40 ratio is a mechanism that might also occur in smooth muscle cells, which in turn could lead to improved vascularisation and could regulate blood pressure. This positive effect on vascularisation and blood pressure might be missing for the AD-typical increased Aβ42 over Aβ40 ratio. This ratio itself, because an inverse Aβ12/Aβ40 ratio led to an inverse regulation of this gene and may not be mediated by unspecific effects occasionally observed in microarray studies.

Taken together, we demonstrated that the expression levels of KLA0125 and NEUROG2 were inversely regulated by an altered Aβ12/Aβ40 ratio: An increased Aβ12/Aβ40 ratio, which is typical of AD, up-regulated NEUROG2 but down-regulated KLA0125, whereas the opposite regulation pattern was observed for a decreased ratio. This might indicate a biological function for the so far uncharacterized KLA0125: it may be speculated that KLA0125 could be involved in neurogenesis possibly by an action antagonistic to that of NEUROG2, due to the observed strict inverse regulation of both genes and the already established involvement of NEUROG2 in developmental neural processes. Finally, our dataset provides information about the regulation of further Aβ dependent genes, which could turn out to be important in AD.

Materials and Methods

Cell line, cell culture and transfections

The human neuroblastoma cell line SH-SY5Y [33,34] was cultured in 50% Minimum Essential Medium (MEM, Sigma) and 50% Nutrient Mixture F-12, HAM (Sigma), supplemented with 10% FBS (PAN), 1% L-Glutamin (Sigma) and 1% nonessential amino acid solution (Sigma) in a humidified atmosphere with 5% CO2. 70% confluent SH-SY5Y cells were transfected with the constructs mentioned below.

Plasmids

Sequences coding for C99WT, C99I45F and C99V50F were cloned into a pCEP4 vector (Invitrogen). These plasmid constructs have been previously described [4,5]. The empty vector pCEP4 (mock) was used as a negative control.

Enzyme-linked immunosorbent assay (ELISA) of Aβ42 and Aβ40

Subconfluent cells were grown in 5 ml culture medium and conditioned for 48 h. Conditioned medium was collected, then Aβ42 and Aβ40 concentrations were measured by an enzyme-linked immunosorbent assay, following the manufacturer’s recommendations. Measurements were carried out using a 96-Well MULTI-SPOT Human (6E10) Abeta Triplex Assay (MSD, Haass).

Transcriptomics and data analysis

Microarray analysis was performed according to the Expression Analysis Technical Manual (Affymetrix) with minor modifications: Briefly, total RNA was extracted using the Qiashredder-Kit, RNase-free DNase set (Qiagen) and RNaseasy Midi-columns (Qiagen). 20 μg of total RNA was reverse transcribed into cDNA by using the SuperscriptTM Double-Stranded cDNA Synthesis Kit (Invitrogen) and oligo(dT) primers (Prolog). 3.3 μl of purified cDNA was converted into cDNA using the BioArrayTM High Yield RNA Labelling Kit (Enzo Life Sciences). Subsequently, 15 μl of purified cDNA was fragmented using the GeneChip® Eukaryotic Hybridization Control Kit (Affymetrix). 15 μg of fragmented cDNA was hybridized to whole genome HG-U133 A and HG-U133 B Chips. Chips were washed, stained, scanned and the quality of the created dat-file images was evaluated by using Gene Operating Software GCOS 1.2 and MAS 5.0 Software (Affymetrix). The sample quality was checked by using a Bioanalyzer 2100 (Agilent). The statistical analysis was carried out using the software package R, version 1.9.1 (R Development
core team (2004): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, together with libraries genma and limma of the Bioconductor project, version 1.4 [35]. The data preprocessing steps, background-adjustment, normalization and computation of GC-RNA gene expression measures were performed according to Wu et al. (Wu Z; Irizarry RA; Gentleman R; Martinez-Murillo F; Spencer F (2004): A Model-Based Background Adjustment for Oligonucleotide Expression Arrays, Johns Hopkins University, Dept. of Biostatistics Working Papers, Working Paper 1). For the statistical analysis, empirical Bayes inference for linear models with the transduced neuroblastoma cell line SH-SY5Y (C99WT1, C99I45F, C99V50F, mock - with 3 replicates per group) was used [36]. Moderated t-statistics and corresponding p-values were calculated for the comparisons C99V50F vs. C99WT1. We used a threshold of 0.005 for the p-values and selected only those probe sets which showed a $\log_{2}$ ratio ≥1. A hierarchical clustering (manhattan metric-complete linkage) for the centered expression profiles over all experimental groups was performed for the presentation of probe sets selected.

Quantitative real-time PCR and selection of an endogenous control for normalisation

Total-RNA was reverse transcribed into cDNA using random hexamer primers included in the High-Capacity cDNA Archive Kit (Applied Biosystems). This cDNA was amplified and measured by using TaqMan® Gene expression assays (Applied Biosystems). Cycling conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative quantification was performed with the $2^{-ACt}$ method. For normalisation, an endogenous control was selected out of 10 candidate controls using the TaqMan® Human Endogenous Control Plate (Applied Biosystems).

Quality control of cells, target-RNA and arrays

Cells were checked for mycoplasma contamination. The 260 nm/280 nm ratio for total-RNA was between 1.9 and 2.1 for microarray experiments. Total-RNA and unfragmented cRNA was checked with a Bioanalyzer 2100 (Agilent). For total-RNA, for microarray experiments. Total-RNA and unfragmented cRNA 260 nm/280 nm ratio for total-RNA was between 1.9 and 2.1

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