Novel AlphasynucleinPAN in human Mesenchymal Stem Cells-derived neurons masks *SNCA140* and creates dissociation of deleterious *SNCA98* in astrocytoma stem cells

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Abstract - Alzheimer's Dementia (AD) and Parkinson's disease Dementia (PDD) are common causes of dementia characterised by misfolded alpha-Synuclein (SNCA) proteins, amyloid precursor proteins (APP), and microtubule associated protein tau (MAPT). Accumulation of alpha synuclein is involved in cancer. *SNCA140* increases oxidative stress, and this leads to elevated amyloidogenic APP processing. There is currently no effective / disease-modifying treatment of AD and PDD. Human mesenchymal stem cells (hMSCs) are promising therapeutic candidates for the repair and regeneration of neural cells, but their viable clinical application in AD and PDD treatment requires consideration of epistatic influence of the lineage substitution of *SNCA*. **Objectives** – Here, we hypothesised that *SNCAPAN* exhibit lineage substitution in hMSCs-derived neurons, and it is epistatic to *SNCA140* expression. **Methodology** – we examined phenotypic characteristic of AD and PDD biomarkers in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene using a Q-PCR. Amplification reactions in *NTCSNCA98* and *NTCSNCA115* were validated by gel electrophoresis. *SNCA98*-*SNCA126* molecular interaction observed was determined using spearman's rank correlation regression, and this was validated by gel electrophoresis of generic PCR (50°C, 52°C, 54°C, 56°C, 58°C, and 60°C) with NTC using Universal human reference RNA (UHRR) and astrocytoma stem cells. Expression of each gene was normalised to 18S, the endogenous control. Data are presented as mean $2^{(-\Delta\Delta Ct)} \pm SEM$. **Results** - *SNCAPAN* exhibits lineage substitution in hMSC-derived neurons, and it is epistatic to *SNCA140* expression, a first time viable novel structural remodelling functional innovation in the treatment of AD, PDD, and Brain Cancer. In *SNCA98* and *SNCA115*, there were NTC amplification reactions. *SNCA98* and *SNCA115* expressions are significantly higher compared to *APP* (P = 0.002947735; P = 1.3411E-07), *MAPT* (P = 0.00184993; P = 5.15474E-05), *SNCAPAN* (P = 0.003579771; P = 3.69932E-06), *SNCA112* (P = 0.006057562; P = 9.06788E-05), and *SNCA126* (P = 0.001054018; P = 2.36E-05) respectively. *SNCA98* expression extremely and significantly correlates with *SNCA126* expression ($\rho = 0.9827$; P = 3.71669E-05). **Conclusion** – The UHRR of gel electrophoresis of generic PCR containing *SNCA126* in astrocytoma stem cells at 50°C and 52°C confirms the significant *SNCA98*-*SNCA126* relationship as sign epistasis (deleterious *SNCA98* enhances beneficial *SNCA126*) due to synergistic epistatic background created by *SNCAPAN*, and this validates the epistasis of *SNCAPAN* to *SNCA140*.

Keywords: *AlphasynucleinPAN*, lineage, hMSCs, *SNCA140*, epistasis, Dementia, astrocytoma

1. Introduction

Dementia is a deterioration progressive cognitive malfunction characterised by inability to perform daily activities with psychiatric symptoms [1]. AD the most common cause of dementia represents 60 - 80% of cases of dementia and is the main cause of morbidity in the developed world [2]. When APP cellular physiological functions neurogenesis, synaptogenesis, and synaptic plasticity fail, APP breakdown

products misfold and lead to amyloid beta (A β) peptide deposition [3]. MAPT encodes Tau protein, which stabilises microtubules, axons and supports cell functions. Tau protein is controlled by phosphorylation and post-translational modifications. In AD brain, higher phosphates aggregate (3 times compared with healthy brain) hyperphosphorylates Tau protein, reducing Tau affinity for microtubules and proteases degradation. This leads to Tau fibrillisation and accumulation into neurofibrillary tangles [4]. AD is characterised by A β deposition, Tau pathology, and neurodegeneration [5-6].

Alpha-synuclein (α -Syn) is a 140 amino acid protein encoded by the SNCA gene [7]. It is 14.5 kDa encoded by 5 exons with total transcripts length of 3041 bps maps on 4q21.3-q22. Lewy bodies the abnormal α -Syn aggregates in neurons [8] can alone cause Dementia with Lewy bodies (DLB) [8] or destroy dopaminergic nerve cells which produces an excitatory neurotransmitter called dopamine, leading to severe neuronal loss in PD. Interestingly, aggregate of Lewy bodies, A β plaque, and tau tangles in the cortex results in PDD [8-9], a characteristic feature of AD. α -Syn increases oxidative stress, which promotes JNK – or NF-KB – mediated BACE 1 transcription, alter subcellular localisation of BASE 1, and thus increases amyloidogenic APP processing [10-12].

In HEK293T cells, canonical *SNCA140* have been reported to exhibit a strong aggregation property compared to the lower aggregation property of alternative spliced minor isoforms *SNCA98*, *SNCA112*, and *SNCA126* [13]. Higher level of *SNCA140* have been reported in AD and PDD patients [14-16]. With therapeutic approaches limited due to adverse side effects and symptom effectiveness, there is currently no effective / disease-modifying treatment of AD and PDD. HMSCs are promising therapeutic candidates for the repair and regeneration of neural cells because of their native expression of significant heparan sulfate proteoglycans core proteins and inherent ability to differentiate towards neural lineages [Rachel Okolicsanyi et al., 2015]. However, their viable clinical application in AD and PDD treatment requires consideration of epistatic influence of the lineage substitution of *SNCA*. Thus, we hypothesised that *SNCAPAN* exhibit lineage substitution in hMSCs-derived neurons, is epistatic to *SNCA140* expression, and create synergistic epistasis in astrocytoma stem cells.

2. MATERIALS AND METHODS

2.1. Preparation of lysates and Q-PCR

Cell pellets of three pooled technical replicate from hMSCs-derived neurons were resuspended TRIzol[®] reagent (cat# 15596026, Invitrogen) and RNA was extracted using the Direct-zol[™] RNA MiniPrep kit (cat#

R2050, Zymo Research) with RNA samples treated in-column with DNase I (cat# R2050, Zymo Research) as described by the manufacturer. For cDNA conversion, 150 ng of RNA sample was incubated with iScript™ reverse transcriptase (cat# 1708891, Bio-rad) according to the manufacturer's instructions. For Q-PCR analysis, we examined phenotypic characteristic of AD and PDD biomarkers in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene: independent variable *SNCAPAN*, dependent variable canonical *SNCA140*, positive controls alternative spliced minor isoforms (*SNCA98*, *SNCA112*, *SNCA115*, *SNCA126*), and negative controls *APP* and *MAPT*. Each reaction mix contained 120 ng of cDNA, 100 Nm of gene-specific forward and reverse primers, 5 µL of SYBR®-Green PCR Master Mix, 0.1 µL of passive reference (ROX) dye (GoTaq® Q-pcr Master Mix, cat# A6001, Promega), and 1.1 µL of nuclease-free H₂O. Amplification was monitored on a QuantStudio™-7 (ThermoFisher Scientific) with cycle conditions consisting of enzyme activation (predenaturation) of 2 minutes at 50 °C and 3 minutes at 95 °C, followed by (denaturation) 50 cycles of 3 seconds at 95 °C, and (Annealing and extension) 30 seconds at 60 °C.

2.2. Validations and statistical analysis

Gel electrophoresis of the q-PCR target genes was conducted to validate the q-PCR amplification reactions in the *NTCSNCA98* and *NTCSNCA115*. In addition, generic PCR of *SNCA98* and *SNCA126* was conducted to validate the observed molecular interaction between *SNCA98* and *SNCA126* at 50°C, 52°C, 54°C, 56°C, 58°C, and 60°C with NTC using Universal human reference RNA (UHRR) and astrocytoma stem cells. This was followed by gel electrophoresis of the generic PCR of the *SNCA98* and *SNCA126*. Expression of each gene was normalised to 18S, the endogenous control. Data are presented as mean $2^{(-\Delta\Delta Ct)} \pm$ SEM. Spearman's rank correlation regression was used to determine the molecular interaction between *SNCA98* and *SNCA126*. Independent student t-test was used to determine relationship between data. Statistical significance was taken as P < 0.05.

3. RESULTS

3.1. Average relative expression of AD and PDD biomarkers in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene (figure 1A).

Figure 1A – Average relative expression of AD and PDD biomarkers in hMSCs-derived neurons at p+6 in four biological replicates ($n = 4$) with non-template controls (NTCs, $n = 2$) for each target gene. There were no amplification reactions in the *NTCAPP*, *NTCMAPT*, *NTCSNCA112*, *NTCSNCA140*, and *NTCSNCAPAN*, but there was amplification reaction in *NTCSNCA98* and *NTCSNCA115*. *SNCA140* was not expressed due to the epistatic influence of *SNCAPAN*. Expression of each gene was normalised to 18S, the endogenous control. Data are presented as mean $2^{(-\Delta\Delta C_t)} \pm SEM$.

To assess epistatic influence of the lineage substitution of *SNCA* in hMSC-derived neurons, *SNCAPAN* along with the positive and negative controls was examined via the Q-PCR. There was no amplification reaction in the *NTCAPP*, *NTCMAPT*, *NTCSNCA112*, *NTCSNCA140*, and *NTCSNCAPAN*, but there was amplification reaction in *NTCSNCA98* and *NTCSNCA115* (Figure 1a). In addition, *SNCA98* demonstrated highest expression level compared to *SNCAPAN*, *SNCA126*, *SNCA115*, *SNCA112*, *MAPT*, and *APP* (Figure 1a). However, the canonical *SNCA140* was not expressed (Figure 1a). Normal human brains have been reported to exhibit lower expression of the alternative spliced minor isoforms *SNCA98*, *SNCA112*, and *SNCA126* compared to the canonical *SNCA140* [13], indicating that *SNCAPAN* expression masks the expression of the canonical *SNCA140* in hMSCs-derived neurons.

Moreover, *SNCA98* and *SNCA115* expressions are significantly higher compared to *APP* ($P = 0.002947735$; $P = 1.3411E-07$), *MAPT* ($P = 0.00184993$; $P = 5.15474E-05$), *SNCAPAN* ($P = 0.003579771$; $P = 3.69932E-06$), *SNCA112* ($P = 0.006057562$; $P = 9.06788E-05$), and *SNCA126* ($P = 0.001054018$; $P = 2.36E-05$) respectively (Figure 1b-c).

Figure 1B – Average relative expression of *SNCA98* and *SNCA115* compared with average relative expression of *APP* and *MAPT* in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. Brown bar: *APP*; Blue bar: *MAPT*; light green: *SNCA98*; Deep green: *SNCA115*; * P < 0.005; ** P < 0.005; *** P < 0.0001.

Figure 1C– Average relative expression of *SNCA98* and *SNCA115* compared with average relative expression of *SNCA98*, *SNCA112*, and *SNCA126* in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. Deep red bar: *SNCA98*; Deep yellow bar: *SNCA112*; Purple bar: *SNCA126*; light green: *SNCA98*; Deep green: *SNCA115*; * P < 0.005; ** P < 0.005; *** P < 0.0001.

We validated our q-PCR results using gel electrophoresis (Figure 2A).

Figure 2A: Gel electrophoresis of the q-PCR target genes using hMSC-derived neurons at p+6 as a template. This experiment was conducted to validate q-PCR amplification reactions in the *NTCSNCA98* and *NTCSNCA115*. L represents DNA ladder with gel bands (DNA fragment) of 100 base pairs each. Three wells were used for each gene, including the NTC. The third well represents the NTC for each gene. There was no gel band in (a) the *NTCAPP*, *NTCMAPT*, *NTCSNCA112*, *NTCSNCAPAN*, *NTC18S* endogenous control (b) all the wells of *SNCA140* (c) the first well of *SNCA98*. Gel bands observed in the *NTCSNCA98* and *NTCSNCA115* validated their q-PCR amplification reactions respectively. The *NTCSNCA126* gel band was suggested to be a molecular interaction (because there was no amplification reaction in the *NTCSNCA126*) with either *SNCA98* or *SNCA115* due to the amplification reactions in the in the *NTCSNCA98* and *NTCSNCA115*. The size of the *NTCSNCA126* gel band was higher compared to the size of the *NTCSNCA98* gel band and *NTCSNCA115* gel band. Furthermore, the intensities of the *NTCSNCA98* gel band and *SNCA98* second well gel band were reduced compared to the gel bands in *SNCA115*. As a group, *SNCA126* gel bands were not travelling together to the same position compared to the gel bands in the *APP*, *MAPT*, *SNCA112*, *SNCA115*, *SNCAPAN*, and the *18S*. Spearman's rank correlation regression showed that molecular interaction is extremely and significantly between *SNCA98* and *SNCA126*. SN or S: *SNCA*; 18S: the endogenous control.

The results shows that there was no gel band in the *NTCAPP*, *NTCMAPT*, *NTCSNCA112*, *NTCSNCAPAN*, *NTC18S* endogenous control, all the wells of *SNCA140*, and the first well of *SNCA98*. Gel bands observed in the *NTCSNCA98* and *NTCSNCA115* validated their q-PCR amplification reactions respectively. The *NTCSNCA126* gel band was suggested to be a molecular interaction (because there was no q-PCR amplification reaction in the *NTCSNCA126*) with either *SNCA98* or *SNCA115* due to the q-PCR amplification reaction and gel band in the in the *NTCSNCA98* and *NTCSNCA115*. The size of the *NTCSNCA126* gel band was higher compared to the size of the *NTCSNCA98* gel band and *NTCSNCA115* gel band. Furthermore, the intensities of the *NTCSNCA98* gel band and *SNCA98* second well gel band were reduced compared to the gel bands in *SNCA115*. As a group, *SNCA126* gel bands were not travelling together to the same position compared to the gel bands in the *APP*, *MAPT*, *SNCA112*, *SNCA115*, *SNCAPAN*, and the *18S*.

We next determined the significance of the observed molecular interaction within *SNCA98*, *SNCA115*, and *SNCA126* using spearman's rank correlation regression (Figure 2b-c).

Figure 2B – Scatter Plot showing an extreme high significant positive correlation between average *SNCA98* expression and *SNCA126* expression in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. ρ : spearman's rank correlation coefficient; P: P-value, significance of the correlation between *SNCA98* and *SNCA126*; R^2 : coefficient of determination.

Figure 2C – Scatter Plot showing a non-significant correlation between average *SNCA115* expression and *SNCA126* expression in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. ρ : spearman's rank correlation coefficient; P: P-value, significance of the correlation between *SNCA115* and *SNCA126*; R^2 : coefficient of determination.

The result shows that *SNCA98* expression extremely and significantly correlates with *SNCA126* expression ($\rho = 0.9827$; $P = 3.71669E-05$) (Figure 2b). However, the correlation between *SNCA115* and *SNCA126* is not statistically significant ($\rho = 0.9819$; $P = 0.9999$) (Figure 2c).

We then validated the observed significant molecular interaction between *SNCA98* and *SNCA126* using gel electrophoresis of the generic PCR product (50°C, 52°C, 54°C, 56°C, 58°C, and 60°C) with NTC, Universal human reference RNA (UHRR), and astrocytoma stem cells (Figure 3).

Figure 3: Gel electrophoresis of generic PCR of *SNCA98* and *SNCA126* exposed to different annealing temperatures (top wells, 50°C-54°C; bottom wells, 56-60°C). This experiment was conducted to validate *SNCA98-SNCA126* molecular interaction. L represents DNA ladder with gel bands of 100 base pairs each. Four wells were used for the gel electrophoresis of the generic PCR per each temperature. For *SNCA98* at each temperature, the first-two wells (II) represent astrocytoma stem cells templates which contain *SNCA98* each respectively. The third well (U) represents UHRR template which contains *SNCA98* and *SNCA126*. The fourth well (N) represents the NTC. For *SNCA126* at each temperature, the first-two wells (II) represent astrocytoma stem cells templates which contain *SNCA126* each respectively. The third well (U) represents UHRR template which contains *SNCA98* and *SNCA126*. The fourth well (N) represents the NTC. Gel electrophoresis of generic PCR product showed that there was no gel band (DNA fragment) in the NTCs across all the temperatures. In addition, there was no gel band in the (a) UHRR template (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA98* each) across all the temperatures (b) UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 54°C, 56°C, 58°C, and 60°C. There were gel bands, each representing same sized DNA fragments in the UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 50°C and 52°C. 98: *SNCA98*; 126: *SNCA126*.

Across all the temperatures, gel electrophoresis of generic PCR product showed that there was no gel band (DNA fragment) in all the NTCs. In addition, there was no gel band in all the UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA98* each) across all the temperatures. Furthermore, there was no gel band in all the UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 54°C, 56°C, 58°C, and 60°C. However, there were gel bands, each representing same size DNA fragments in the UHRR templates (third well that

contains *SNCA98* and *SNCA126*) next to the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 50°C and 52°C.

4. DISCUSSION

SNCA (encodes a 140 amino acid protein called Alpha-synuclein, α -Syn) is one of the main causative genes in neurodegenerative diseases. Lineage specific substitutions occurred during evolution have been mapped on human *SNCA* with the help of ancestor reconstruction technique [17]. α -Syn increases oxidative stress, which promotes JNK – or NF-KB – mediated BACE 1 transcription, alter subcellular localisation of BACE 1, and thus increases amyloidogenic APP processing [10-12]. Oxidative stress is implicated in AD and PDD, and other neurodegenerative diseases. Higher level of intracellular reactive oxygen species (ROS) can cause DNA and protein damage leading to cellular death (). ROS can also trigger mitochondrial and endoplasmic reticulum stress death pathways.

Pan troglodytes (Chimpanzee) *SNCA*, *SNCAPAN*, is a neighbor to homo sapiens (human) *SNCA* in evolutionary relationship of the *SNCA* family members [17]. Viable clinical application of hMSCs in AD and PDD treatment requires consideration of epistatic influence of the lineage substitution of *SNCA*. In our study, *SNCAPAN* exhibits lineage substitution in hMSC-derived neurons, and it is epistatic to *SNCA140* expression, a first time viable novel structural remodeling functional innovation in the treatment of AD, DLB, PDD, and cancer. Gel electrophoresis of the q-PCR products validated the epistasis of *SNCAPAN* to *SNCA140* expression. According to Bungeroth et al., 2014, higher level of *SNCA140* is expressed in normal human brain [13] and Higher level of *SNCA140* have been reported in AD and PDD patients [14-16].

Low expression (1% - 3% of the canonical *SNCA140*) of the alternative spliced minor isoforms *SNCA126*, *SNCA112*, and *SNCA98* have been reported in normal brain [13]. Owing to the amplification reactions in the *NTCSNCA98*, *SNCA98* expressions is significantly highest compared to the expressions of other the positive controls and the negative controls. This is contrary to the lowest expression of *SNCA98* in the total brain, Cortex, Nucleus Caudatus, Putamen, and Substantia Nigra [13]. Furthermore, compared to the low total brain expression of *SNCA112* in normal brain, the level of *SNCA112* in our study is lower. The expression of the remaining positive controls alternative spliced minor isoforms and the negative controls are not consistently higher than each other except for *SNCA115*. The amplification reactions in the *NTCSNCA115* results in a significantly higher *SNCA115* expression compared to the other positive controls and the negative controls.

SNCA115 transcript variants is associated with α -Syn protein aggregation. The *SNCA115* transcript variant encodes 115-amino acid peptide. However, this transcript variant is highly associated with alcoholism [7]. This could be the reason there is non-significant correlation between average *SNCA115* expression and *SNCA126* expression in our result. In summary, our investigation opens the door for further structural remodelling functional studies of *SNCAPAN* in AD, PDD, other neurodegenerative diseases, and cancer.

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Figure 1A – Average relative expression of AD and PDD biomarkers in hMSCs-derived neurons at p+6 in four biological replicates ($n = 4$) with non-template controls (NTCs, $n = 2$) for each target gene. There were no amplification reactions in the *NTCAPP*, *NTCMAPT*, *NTCSNCA112*, *NTCSNCA140*, and *NTCSNCAPAN*, but there was amplification reaction in *NTCSNCA98* and *NTCSNCA115*. *SNCA140* was not expressed due to the epistatic influence of *SNCAPAN*. Expression of each gene was normalised to 18S, the endogenous control. Data are presented as mean $2^{-\Delta\Delta C_t} \pm$ SEM.

Figure 1B – Average relative expression of *SNCA98* and *SNCA115* compared with average relative expression of *APP* and *MAPT* in hMSCs-derived neurons at p+6 in four biological replicates ($n = 4$) with non-template controls (NTCs, $n = 2$) for each target gene. Brown bar: *APP*; Blue bar: *MAPT*; light green: *SNCA98*; Deep green: *SNCA115*; * $P < 0.005$; ** $P < 0.005$; *** $P < 0.0001$.

Figure 2B – Scatter Plot showing an extreme high significant positive correlation between average SNCA98 expression and SNCA126 expression in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. p : spearman's rank correlation coefficient; P : P-value, significance of the correlation between SNCA98 and SNCA126; R^2 : coefficient of determination.

Figure 2C – Scatter Plot showing a non-significant correlation between average SNCA115 expression and SNCA126 expression in hMSCs-derived neurons at p+6 in four biological replicates (n = 4) with non-template controls (NTCs, n = 2) for each target gene. p : spearman's rank correlation coefficient; P : P-value, significance of the correlation between SNCA115 and SNCA126; R^2 : coefficient of determination.

Figure 3: Gel electrophoresis of generic PCR of *SNCA98* and *SNCA126* exposed to different annealing temperatures (top wells, 50°C-54°C; bottom wells, 56-60°C). This experiment was conducted to validate *SNCA98-SNCA126* molecular interaction. L represents DNA ladder with gel bands of 100 base pairs each. Four wells were used for the gel electrophoresis of the generic PCR per each temperature. For *SNCA98* at each temperature, the first-two wells (II) represent astrocytoma stem cells templates which contain *SNCA98* each respectively. The third well (U) represents UHRR template which contains *SNCA98* and *SNCA126*. The fourth well (N) represents the NTC. For *SNCA126* at each temperature, the first-two wells (II) represent astrocytoma stem cells templates which contain *SNCA126* each respectively. The third well (U) represents UHRR template which contains *SNCA98* and *SNCA126*. The fourth well (N) represents the NTC. Gel electrophoresis of generic PCR product showed that there was no gel band (DNA fragment) in the NTCs across all the temperatures. In addition, there was no gel band in the (a) UHRR template (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA98* each) across all the temperatures (b) UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to all the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 54°C, 56°C, 58°C, and 60°C. There were gel bands, each representing same sized DNA fragments in the UHRR templates (third well that contains *SNCA98* and *SNCA126*) next to the astrocytoma stem cells templates (first two wells that contain only *SNCA126* each) at 50°C and 52°C. 98: *SNCA98*; 126: *SNCA126*.