



An Isogenic Collection of Pluripotent Stem Cell Lines With Elevated α -Synuclein Expression Validated for Neural Induction and Cortical Neuron Differentiation

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α -Synuclein (α Syn) is a small, disordered protein that becomes aggregated in Lewy body diseases, such as Parkinson's disease (PD) and dementia with Lewy bodies (DLB). Human induced pluripotent stem cells (hiPSCs) potentially provide a tractable disease model to monitor early molecular changes associated with PD/DLB. We and others have previously derived hiPSC lines from patients with duplication and triplication of the *SNCA* gene, encoding for α Syn. It is now recognised that to perform meaningful disease modelling with these hiPSC lines, it is critical to generate isogenic control cell lines that lack the disease causing mutations. In order to complement the existing and emerging hiPSC models for PD/DLB, we have generated an allelic series of α Syn over-expressing hESC lines on the same isogenic background. An unresolved question is whether pluripotent stem cell lines, with elevated levels of α Syn, can undergo efficient differentiation into dopaminergic and cortical neurons to model PD and DLB, respectively. We took advantage of our isogenic collection of hESC lines to determine if increased expression of α Syn affects neural induction and neuronal differentiation. Clonal hESC lines with significantly different levels of α Syn expression proliferated normally and maintained expression of pluripotent markers, such as OCT4. All cell lines efficiently produced PAX6⁺ neuroectoderm and there was no correlation between α Syn expression and neural induction efficiency. Finally, global transcriptomic analysis of cortical differentiation of hESC lines with low or high levels of α Syn expression demonstrated robust and similar induction of cortical neuronal expression profiles. Gene expression differences observed were unrelated to neural induction and neuronal differentiation. We conclude that elevated expression of α Syn in human pluripotent stem cells does not adversely affect their neuronal differentiation potential and that collections of isogenic cell lines with differing levels of α Syn expression are valid and suitable models to investigate synucleinopathies.

Keywords: human pluripotent stem cells, α -synuclein, synucleinopathy, isogenic cell lines, cortical differentiation, neurogenesis, Parkinson's disease

INTRODUCTION

Multiple lines of evidence have implicated α Syn as a major pathological driver in PD (Polymeropoulos et al., 1997; Spillantini et al., 1997; Singleton et al., 2003; Chartier-Harlin et al., 2004). The genetic forms of PD have led to the development of patient-derived and engineered pluripotent stem cell models to gain mechanistic insights into synucleinopathies (Singh Dolt et al., 2017). A key question for the validity of such models is whether mutant or elevated α Syn expression disrupts early neural induction or neuronal differentiation, thereby limiting later phenotypic analysis and disease modelling. Current data in the literature is conflicting, with some studies proposing that high levels of α Syn alter cell fate and differentiation (Schneider et al., 2007; Oliveira et al., 2015), whereas others have found no evidence for impaired neurogenesis (Devine et al., 2011; Prots et al., 2018; Brazdis et al., 2020). Furthermore, α Syn is known to bind DNA and alter expression of other genes, however the exact mechanism of this affect is not known (Surguchev and Surguchov, 2017). In this study, we aimed to generate an isogenic collection of hESC lines with differing levels of α Syn expression and use this to systematically investigate if elevated α Syn expression affects cortical neuron differentiation. If either the efficiency of neural induction or fidelity of neuronal differentiation is affected, then this may be a source of bias in studies comparing cultured neurons with differential α Syn expression. Isogenic cellular models provide the best experimental system to test this directly.

Studies utilising α Syn null mouse models showed no major differences in neuronal development and overall brain structure (Abeliovich et al., 2000; Specht and Schoepfer, 2001; Gretchen-Harrison et al., 2010). However, subtle differences in dopaminergic neurotransmission were reported and these were age-dependent (Al-Wandi et al., 2010; Anwar et al., 2011; Connor-Robson et al., 2016). Rodent models employing transgenic over-expression of wild-type α Syn have reported impaired adult neurogenesis, but have not determined whether this is a neurodevelopmental defect from birth or a toxic effect of α Syn over-expression (Winner et al., 2012). We have previously shown that hiPSCs with an SNCA triplication mutation have a two-fold increase in α Syn protein levels, and this did not significantly impair dopaminergic neuronal differentiation (Devine et al., 2011). This was also shown by other independent groups using hiPSCs harbouring the SNCA triplication (Byers et al., 2011; Lin et al., 2016) and SNCA duplication mutations (Prots et al., 2018; Brazdis et al., 2020). However, other studies using SNCA triplication mutation-derived hiPSCs reported reduced neuronal differentiation capacity and impaired neurite outgrowth (Flierl et al., 2014; Oliveira et al., 2015). Furthermore, other studies using lentiviral-mediated Dox-inducible expression of α Syn, in human iPSC-derived neuronal progenitors, reported altered cell fate and impaired differentiation of neural stem cells into neurons (Schneider et al., 2007; Zasso et al., 2018). The cellular and rodent data described so far report conflicting roles for α Syn in neurogenesis, highlighting the need for work to clarify this point.

In this study, we generated an allelic series of clonal isogenic hESC lines expressing a broad range of α Syn, including lines expressing the protein at supraphysiological levels. We then set

out to determine whether elevated α Syn expression impairs cortical neuron differentiation of human pluripotent stem cells. We show, using marker analysis during multiple rounds of differentiation and unbiased transcriptomic analysis, that cortical neuron differentiation is not impaired by increased expression of α Syn. The strength of our approach is the use of an isogenic collection of hESC lines with differing and sustained α Syn expression during differentiation, robust cortical neuron differentiation protocols and unbiased analysis of the transcriptome before and after differentiation. This study provides a valuable collection of cell lines for the neuroscience community and provides important evidence for the validity of hESC/iPSC disease models with elevated levels of α Syn expression.

RESULTS

Generation of Clonal Isogenic hESC Lines With Differing Levels of α Syn Expression

An allelic series of clonal transgenic hESC lines was established using a human SNCA cDNA expression cassette driven by the pCAG promoter (Figure 1A), reported to maintain stable and ubiquitous transgene expression across diverse cell types (Hitoshi et al., 1991; Liew et al., 2007). Multiple puromycin-resistant clones were established on a parental Shef4 hESC line and examined for SNCA expression using qRT-PCR (Figure 1B). In an undifferentiated state, 11 clones (S5, S7, S9, S10, S12, S13, S17, S20, S21, S22, and S34) had similar SNCA expression to Shef4, whereas 18 clones (S2, S4, S6, S8, S11, S14, S15, S16, S18, S19, S23, S24, S30, S32, S33, S35, S36, and S37) had elevated expression of SNCA (4-fold–30-fold) relative to the parental cell line (Figure 1B). V2 and V39 control lines, transfected with a control plasmid lacking SNCA, expressed similar levels of SNCA to the parental cell line, Shef4. Clones were selected based on stability of SNCA mRNA expression and placed into low (Shef4, S9, S34) and high (S8, S37) groups. Each of these lines similarly expressed the pluripotency marker, OCT4, and maintained hESC morphology in their undifferentiated state (Figure 1C). Elevated expression of α Syn protein was confirmed for selected clones using immunohistochemistry and western blotting (Figures 1C,D).

The impact of α Syn on cell proliferation was assessed using an MTS assay. Over a 10-day period, six low α Syn lines (S9, S12, S13, S17, S22, S34) and 3 high α Syn lines (S8, S36, S37) were examined and each showed a similar rate of proliferation (Supplementary Figure S1). There was no significant difference in the proliferation rate of low and high α Syn groups and linear regression analysis showed no correlation between SNCA expression and proliferation rate of the examined cell lines ($R^2 = 0.003$, $p = 0.884$).

Elevated α Syn Expression in hESC Lines Is Compatible With Cortical Neuronal Differentiation

Multiple clonal lines were differentiated into cortical neural progenitors using a dual SMAD inhibition protocol (Figure 2A) (Chambers et al., 2009; Shi et al., 2012). RNA

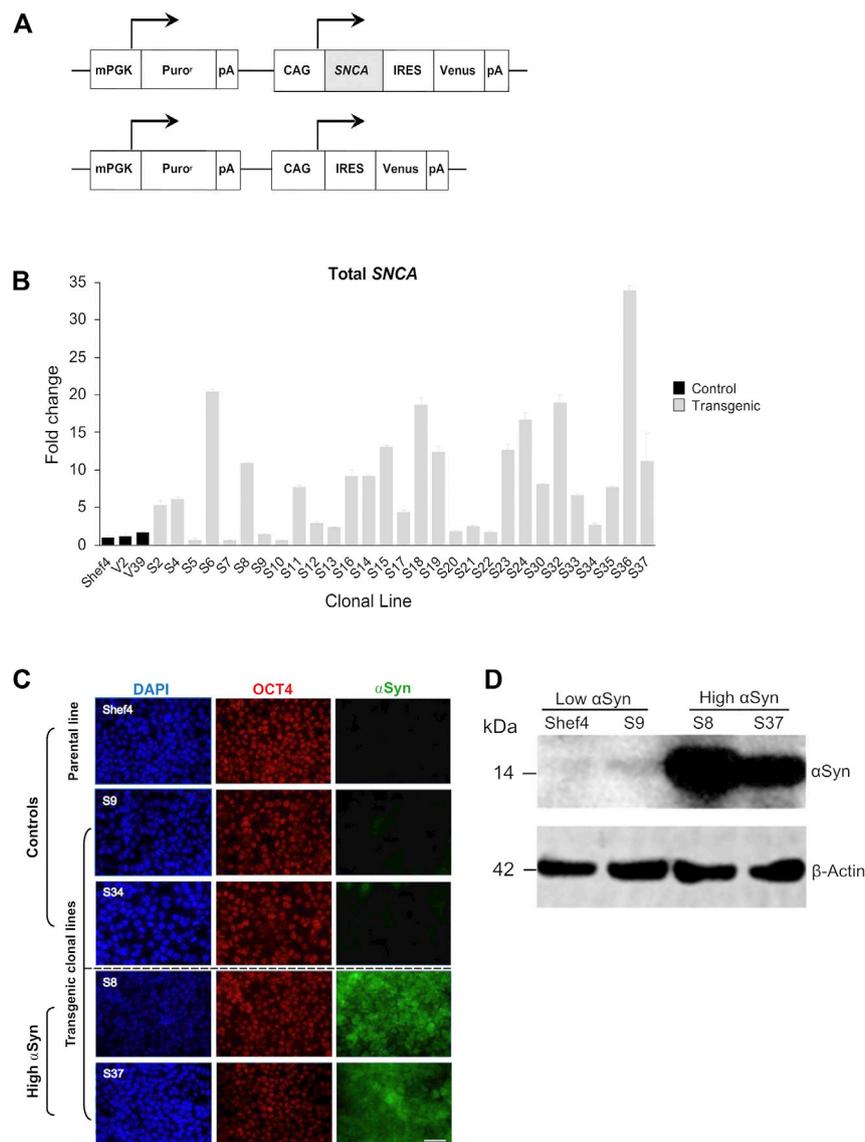
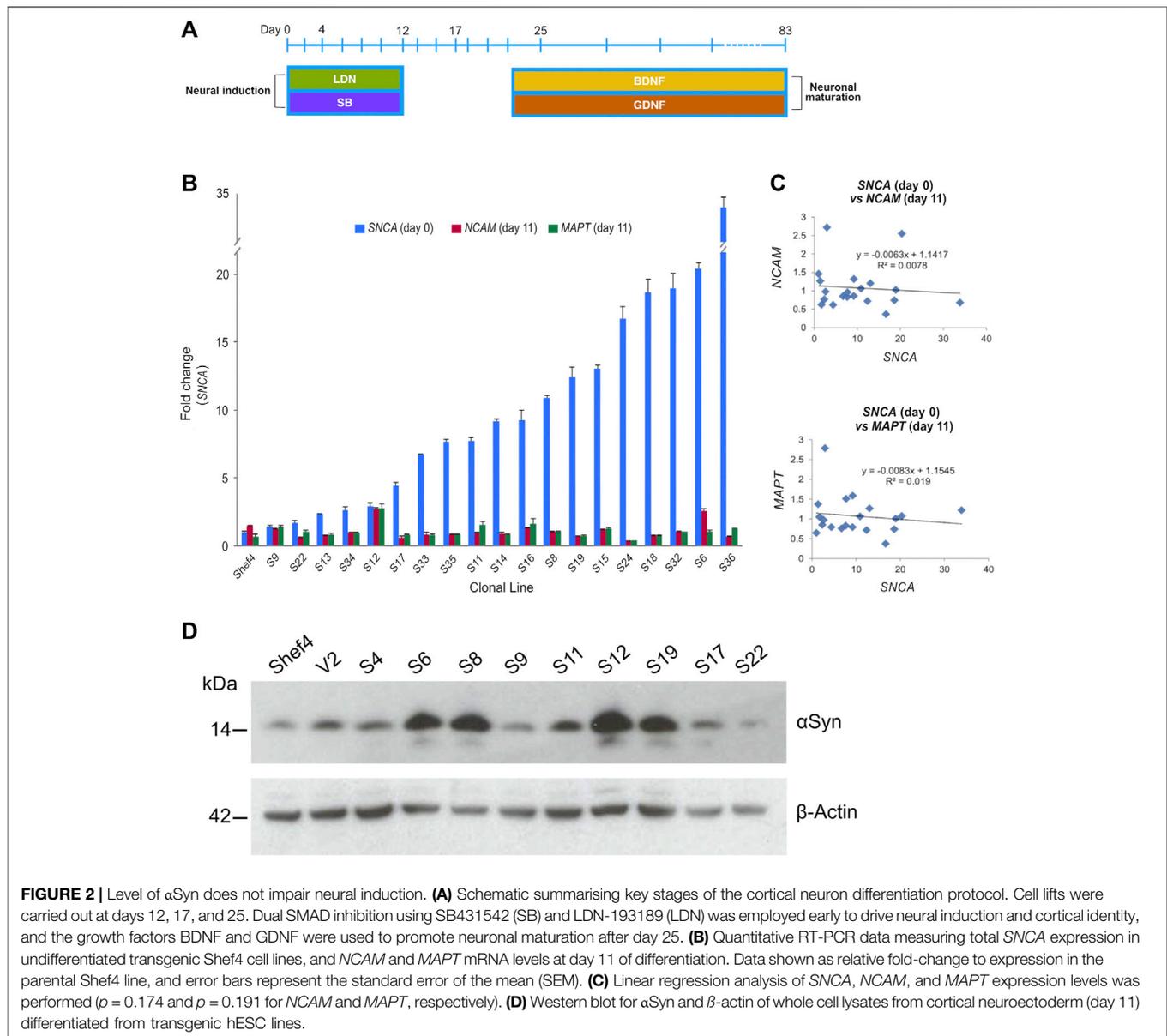


FIGURE 1 | Establishment of clonal hESC lines over-expressing α Syn. **(A)** Schematic of pCAG-SNCA-IRES-Venus and pCAG-IRES-Venus constructs transfected into Shef4 hESCs to generate clonal lines. **(B)** Quantitative RT-PCR measuring total SNCA expression (mRNA) levels in multiple self-renewing undifferentiated transgenic clones generated from parental Shef4 hESC lines. Data was normalized to 18S rRNA levels, and shown relative to expression in the parental hESC Shef4 line. Each bar represents the mean and standard deviation of three technical replicates. **(C)** Representative immunocytochemistry images of undifferentiated hESC clones derived from the parental cell line Shef4, and transgenic clonal lines S9, S34, S8, and S37, stained for DAPI (blue), OCT4 (red), and α Syn (green). Scale bar = 50 μ m. **(D)** Western blot for total α Syn and β -actin in undifferentiated clonal lines for Shef4 and S9 (low α Syn), and S8 and S37 (high α Syn) clones.

was isolated and qRT-PCR used to quantify *NCAM* and *MAPT* expression at day 11 and compared to *SNCA* expression at the start of differentiation (**Figure 2B**). There was no correlation in day 11 *NCAM* ($R^2 = 0.008$, $p = 0.761$) nor day 11 *MAPT* ($R^2 = 0.019$, $p = 0.461$) levels relative to *SNCA* expression for the clonal lines (**Figure 2C**). Western blotting at day 11 of differentiation also confirmed differential protein expression of α Syn in low and high α Syn cell lines (**Figure 2D**). hESC lines with low or high α Syn expression differentiated equally well to form PAX6-positive neuroectoderm by day 12 (**Figure 3A**). A direct comparison

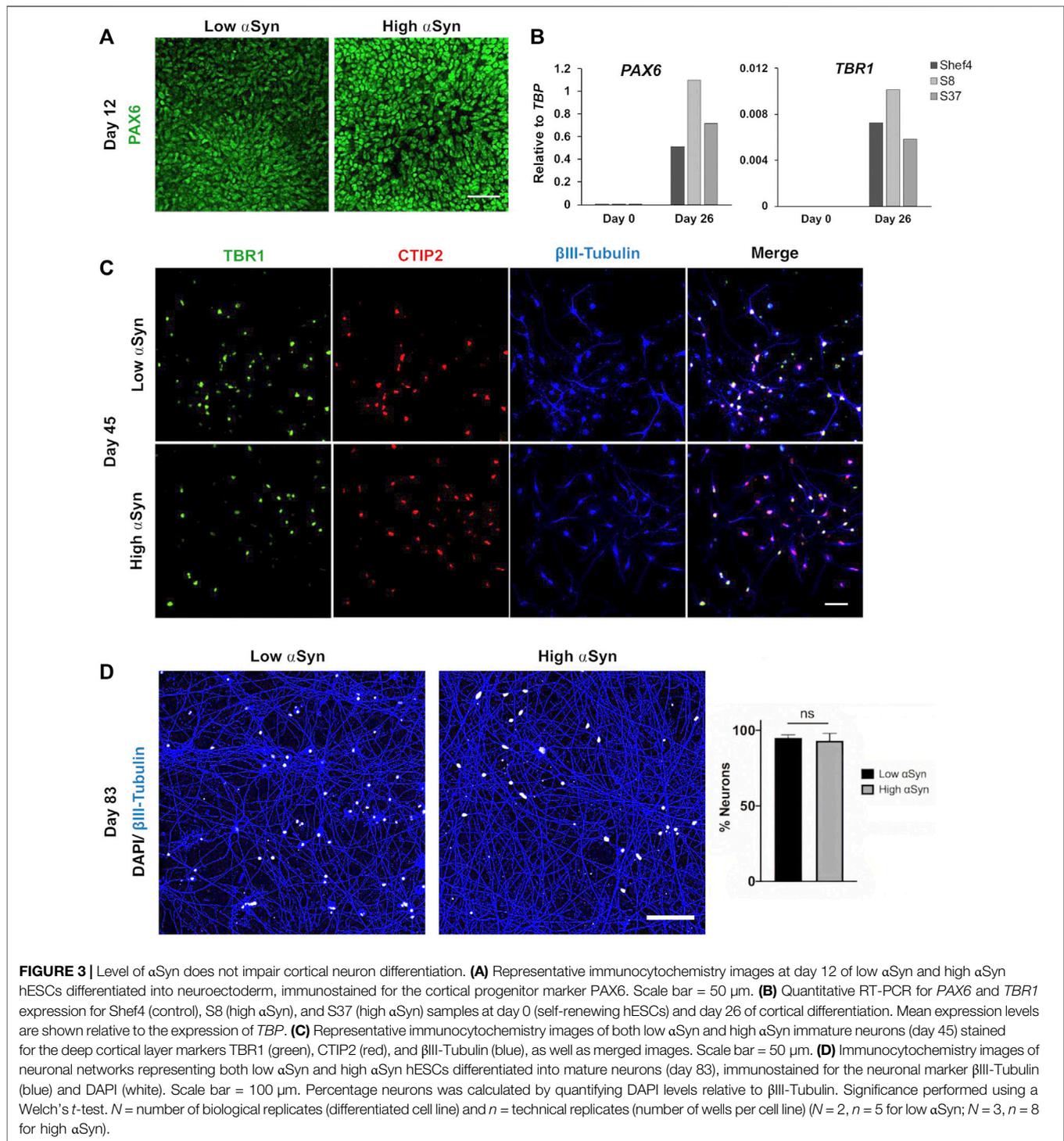
of Shef4 and selected clonal hESC lines with human iPSC lines from a healthy control (NAS2) and triplication *SNCA* patient (AST18) showed robust and similar expression of cortical progenitor markers PAX6, OTX1/2, and Vimentin at day 12 of neural induction (**Supplementary Figure S2**). By day 26 of cortical neuron differentiation the mRNA expression of PAX6 and the cortical marker, *TBR1*, were similarly induced in the parental Shef4 cell line and two high α Syn clones (S8, S37) (**Figure 3B**). Furthermore, by day 45, there was similar protein expression of early cortical markers, TBR1 and CTIP2, in low and high α Syn groups (**Figure 3C**). Despite the difference in



α Syn levels, both groups formed neuronal networks with the same degree of efficiency based on β III-Tubulin immunostaining (Figure 3D). This data provides evidence that α Syn expression levels do not influence neuronal induction and differentiation potential across multiple clonal hESC lines.

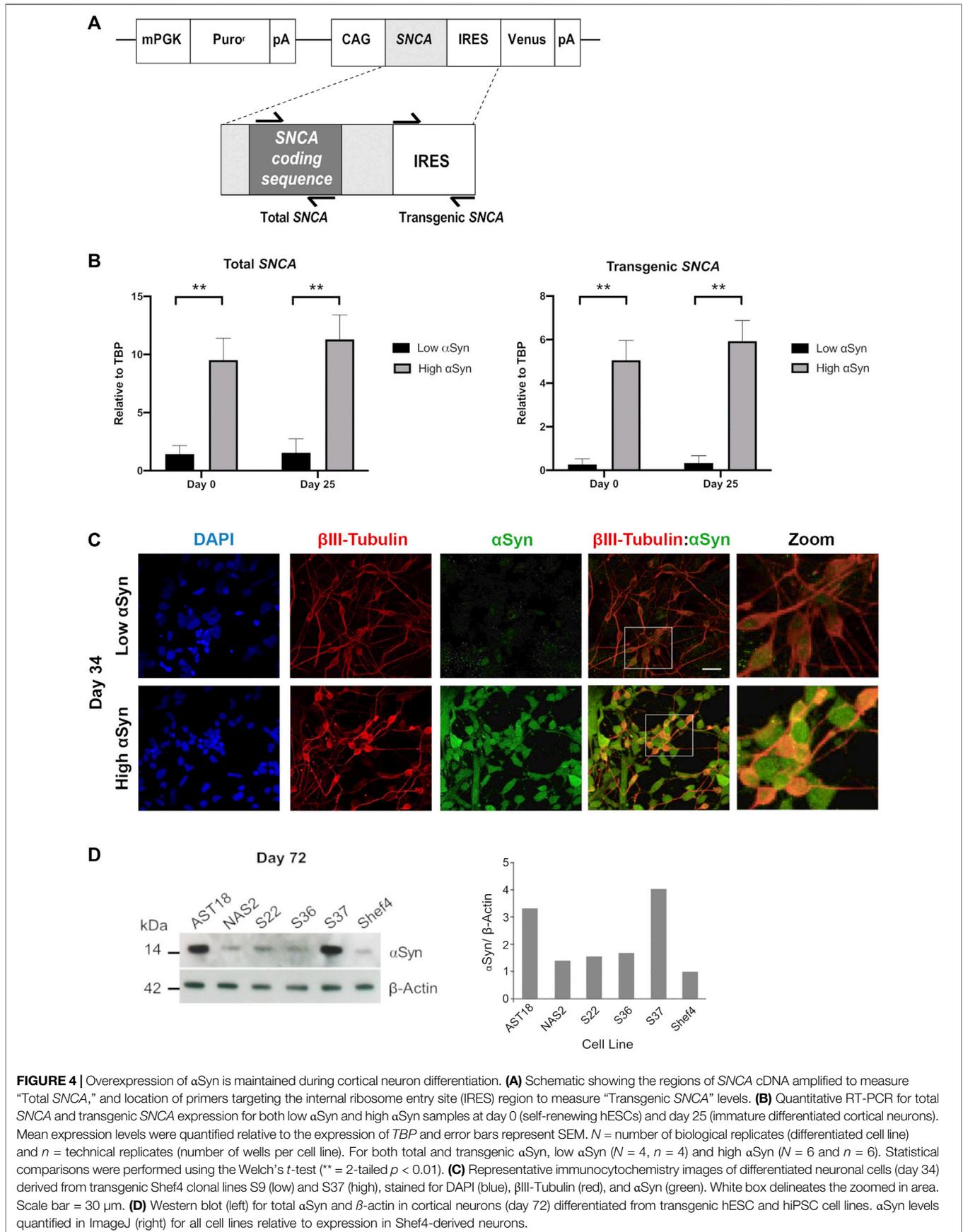
To determine if transgenic *SNCA* expression was maintained during differentiation, total and transgenic *SNCA* levels were measured by qRT-PCR (Figure 4A). The primers for total *SNCA* were designed to target the coding region and primers for transgenic *SNCA* targeted the IRES region (Figure 4A). Total and transgenic levels of human *SNCA* were measured in self-renewing hESCs (day 0) and day 25 differentiated cortical cells. Day 25 was a suitable time point as the neural induction period has been completed and immature cortical neurons are forming.

Total *SNCA* expression at day 0 was significantly higher in the high α Syn vs. low α Syn hESC group (Figure 4B). Importantly, at day 25, the elevated expression of *SNCA* was maintained between the high α Syn vs. low α Syn cortical neuron group ($p < 0.01$). Total *SNCA* levels did not significantly increase over each time point, day 0 vs. day 25, for both high α Syn and low α Syn groups. Transgenic *SNCA* levels in undifferentiated hESCs were significantly higher in the high α Syn vs. low α Syn group (Figure 4B) ($p < 0.01$). Day 25 high α Syn vs. low α Syn cortical neurons also had higher transgenic *SNCA* ($p < 0.01$). Transgenic *SNCA* levels did not significantly increase or decrease between day 0 and day 25 for the high α Syn and low α Syn groups. Immunostaining and western blotting confirmed that high α Syn expression was maintained following cortical neuron differentiation (Figures 4C,D). At day 72 of cortical



differentiation the hiPSC line, AST18, containing an SNCA triplication mutation exhibited a 3-fold increase in α Syn expression compared to a control hiPSC line, NAS2, derived from a 1st-degree relative (Devine et al., 2011) (Figure 4D). The S37 transgenic cell line maintained high α Syn expression similar to AST18 neurons, while the S36 hESC line, that expressed the most SNCA at day 0

(Figure 1B), now had low α Syn expression by day 72 of differentiation, suggesting significant transgene silencing (Figure 4D). This is not unexpected as transgene expression is contingent on several factors, including site of integration, number of copies of the transgene, as well as transgene silencing over cell passage or differentiation (Liu et al., 2009).



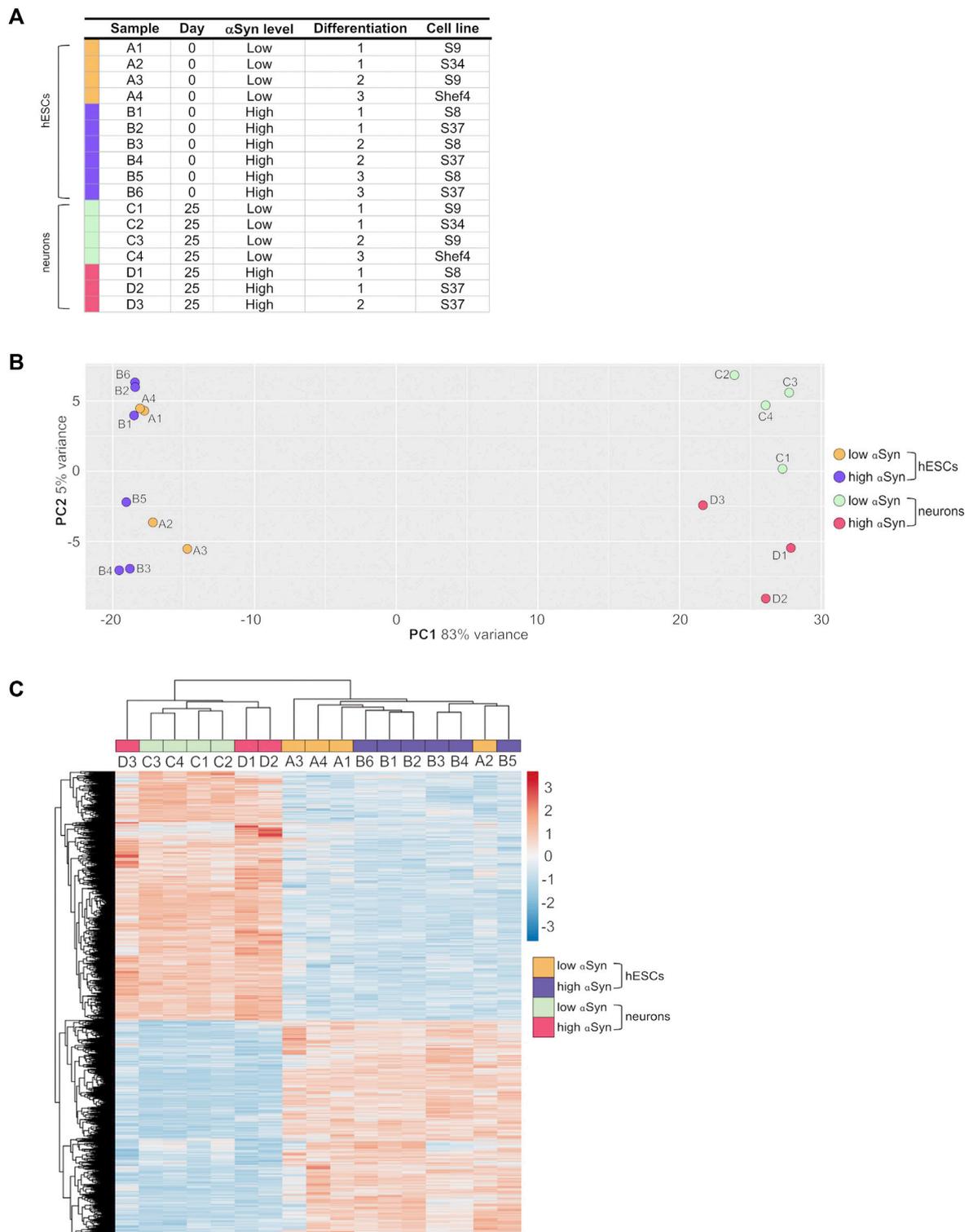


FIGURE 5 | RNA-seq analysis of clonal hESC lines and differentiated cortical neurons. **(A)** Table of individual sample groups highlighting α Syn levels, differentiation experiment group, and the cell line of samples. $N = 3$ for high α Syn cortical neurons, $N = 4$ for both low α Syn hESCs and cortical neurons, and $N = 6$ for high α Syn hESCs. **(B)** Principal component analysis (PCA) plot of all samples. **(C)** Heatmap of a hierarchical cluster analysis of differential expression results, showing relative gene expression changes as either upregulated (red) or downregulated (blue). Analysis was carried out with \log_2 -transformed raw counts. Plot encompasses all differentially expressed genes from all four DESeq2 pairwise comparisons, representing 5,157 genes in total.

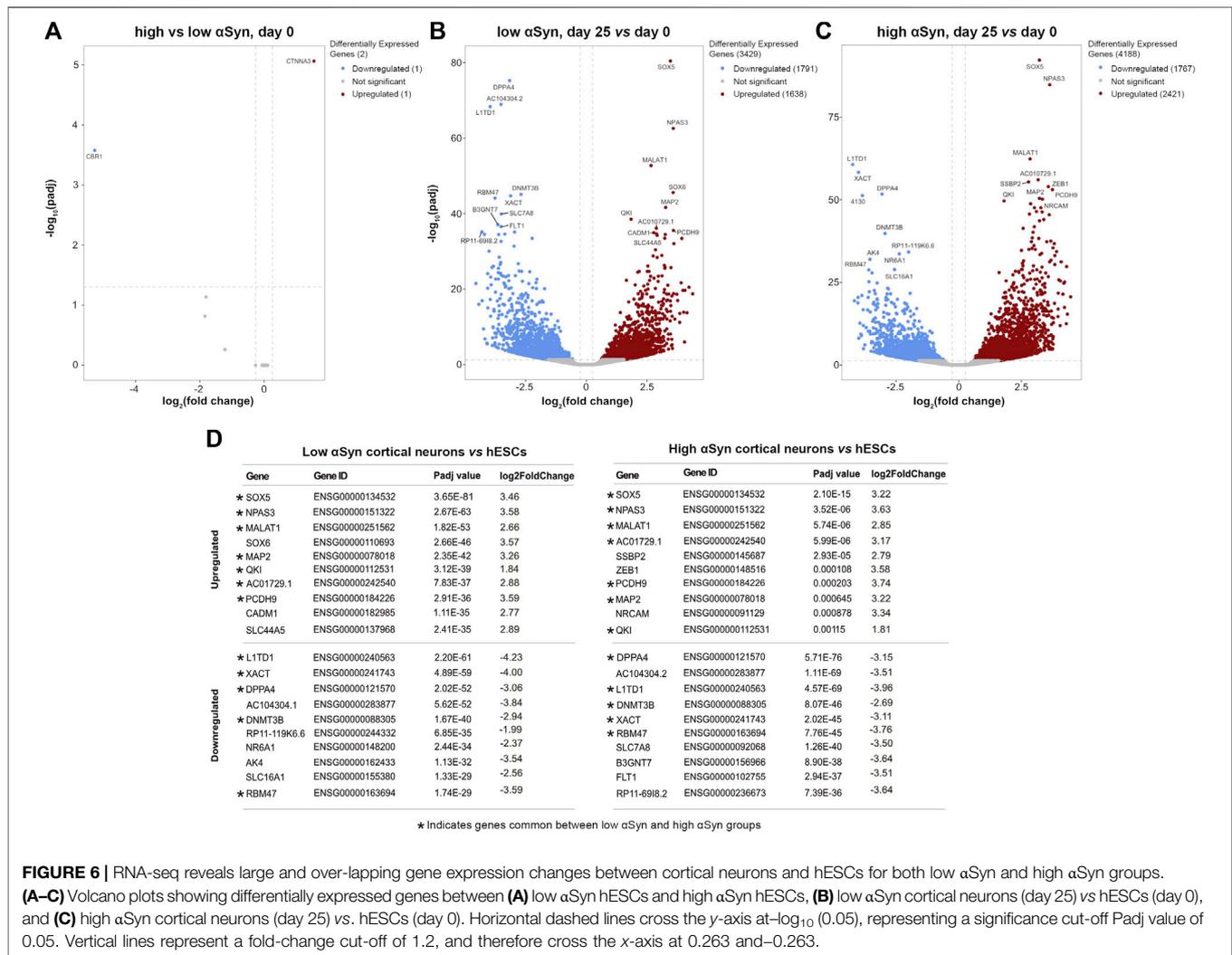


FIGURE 6 | RNA-seq reveals large and over-lapping gene expression changes between cortical neurons and hESCs for both low α Syn and high α Syn groups. (A–C) Volcano plots showing differentially expressed genes between (A) low α Syn hESCs and high α Syn hESCs, (B) low α Syn cortical neurons (day 25) vs. hESCs (day 0), and (C) high α Syn cortical neurons (day 25) vs. hESCs (day 0). Horizontal dashed lines cross the y-axis at $-\log_{10}(0.05)$, representing a significance cut-off Padj value of 0.05. Vertical lines represent a fold-change cut-off of 1.2, and therefore cross the x-axis at 0.263 and -0.263 .

α Syn Over-Expression During Cortical Differentiation Does not Alter the Transcriptional Signature Associated with Neurogenesis

The transcriptomic profile of self-renewing hESC lines (day 0) and differentiated cortical neurons (day 25) with high α Syn and low α Syn were investigated using RNA-seq analysis. A total of 17 samples were subjected to UPX 3'-sequencing to measure polyadenylated mRNA transcripts across 4 sample groups 1) low α Syn hESCs, 2) high α Syn hESCs, 3) low α Syn cortical neurons, and 4) high α Syn cortical neurons (Figure 5A). Principal Component Analysis (PCA) showed a clear segregation between hESCs and cortical neurons, represented by 83% variance on PC1 (Figure 5B). The PC2 axis was representative of α Syn-related differences between hESC and cortical neuron samples. The hESC samples were intermixed, but cortical neuron samples segregated based on α Syn expression, however, the variance along PC2 was small (5%). This is further corroborated by hierarchical clustering analysis based on all

differentially expressed genes across the four sample groups (Figure 5C).

A pairwise comparison of self-renewing high α Syn vs low α Syn hESC lines found only 2 genes (*CBR1*, *CTNNA3*) were significantly differentially expressed between the two groups (Figure 6A), indicating α Syn does not impact on the pluripotency transcriptome. As expected, comparisons of low α Syn cortical neurons and low α Syn hESCs, as well as high α Syn cortical neurons and high α Syn hESCs, identified large numbers of differentially expressed genes (3,429 and 4,188, respectively, Figures 6B,C; Supplementary Table S1). Seven of the top ten most significantly upregulated genes were the same for these two pairwise comparisons, including *SOX5*, *NPAS3*, *MALAT1*, *MAP2*, *QKI*, *PCDH9*, and *AC10729.1* (Figure 6D). Most of these genes have a role in neurogenesis (Izant and McIntosh, 1980; Hardy et al., 1996; Wunderle et al., 1996; Strehl et al., 1998; Brunskill et al., 1999; Chen et al., 2016). Similarly, five of the top ten significantly downregulated genes were the same in the hESCs vs cortical neuron comparisons for high and low α Syn cell lines, including *DPPA4*, *L1TD1*, *RBM47*, *XACT* and *DNMT3B*

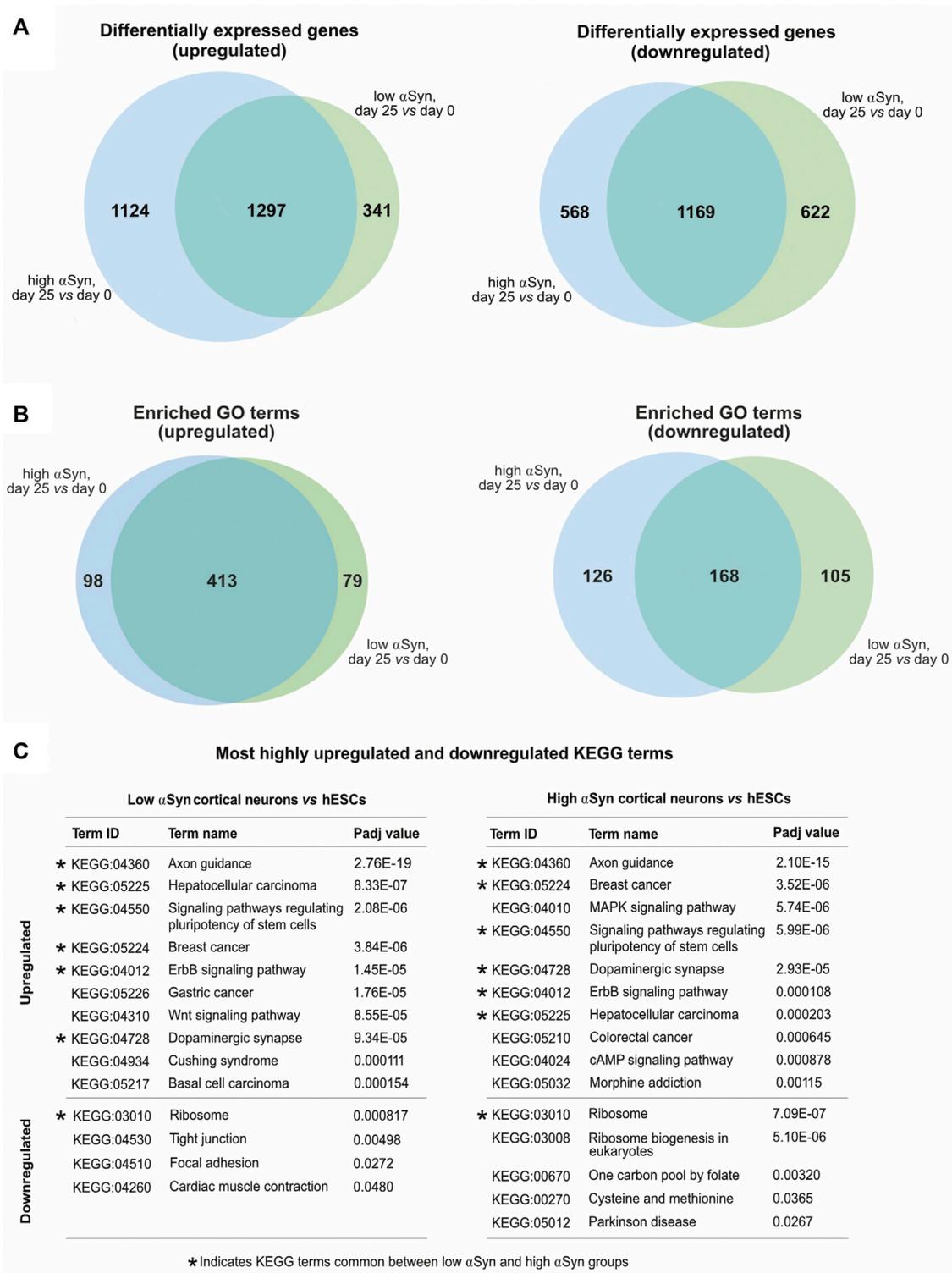


FIGURE 7 | (A) Venn diagrams comparing upregulated and downregulated differentially expressed genes from low α Syn cortical neurons vs hESCs and high α Syn cortical neurons vs hESCs comparisons. **(B)** Venn diagrams comparing upregulated and downregulated GO terms from low α Syn cortical neurons vs hESCs and high α Syn cortical neurons vs hESCs comparisons. **(C)** List of top 10 upregulated and top 10 downregulated KEGG terms by Padj value, in order of ascending Padj, for low α Syn cortical neurons vs hESCs and high α Syn cortical neurons vs hESCs (if less than 10, all terms shown). KEGG terms common to both comparisons are indicated.

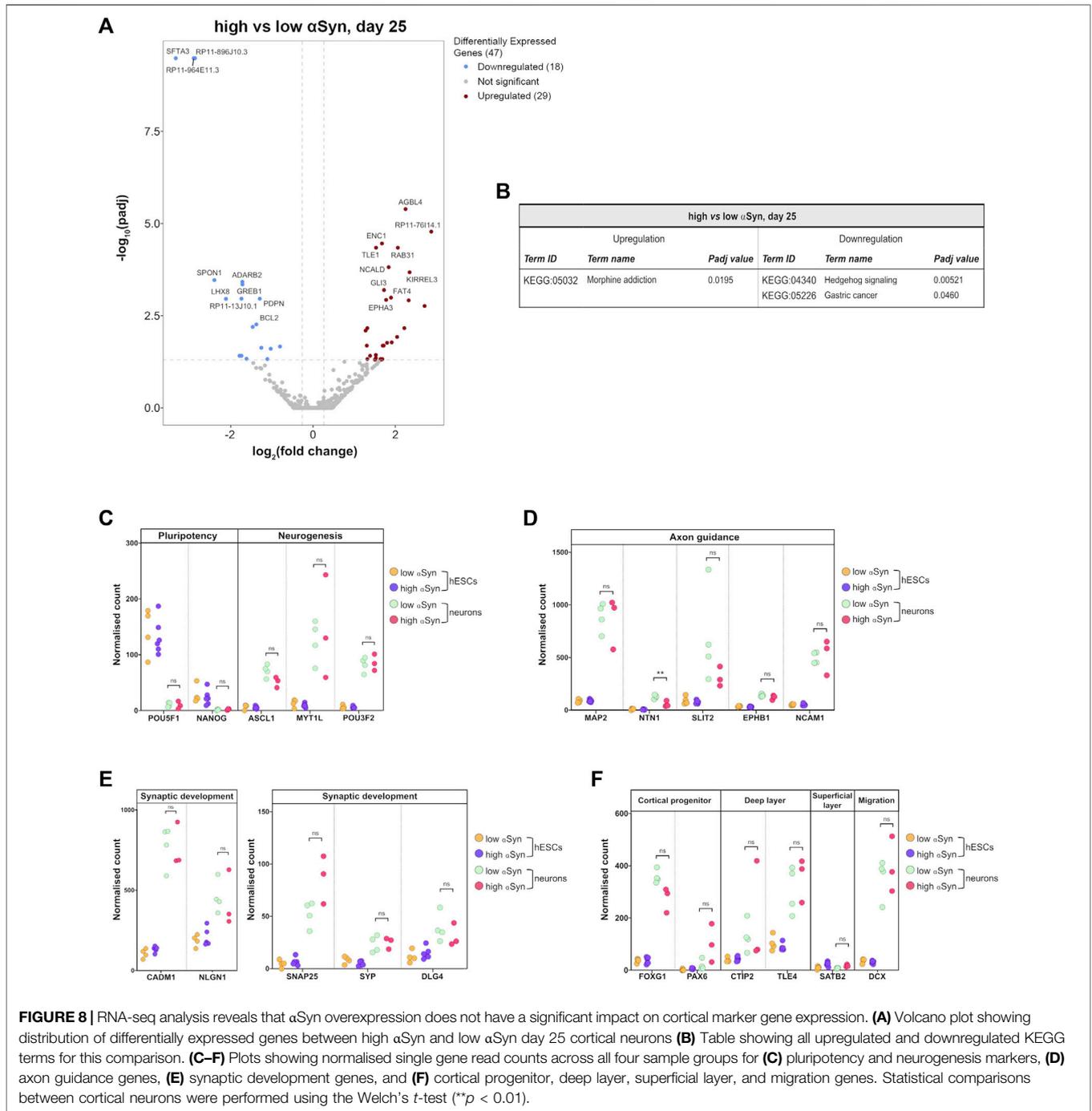


FIGURE 8 | RNA-seq analysis reveals that α Syn overexpression does not have a significant impact on cortical marker gene expression. **(A)** Volcano plot showing distribution of differentially expressed genes between high α Syn and low α Syn day 25 cortical neurons **(B)** Table showing all upregulated and downregulated KEGG terms for this comparison. **(C–F)** Plots showing normalised single gene read counts across all four sample groups for **(C)** pluripotency and neurogenesis markers, **(D)** axon guidance genes, **(E)** synaptic development genes, and **(F)** cortical progenitor, deep layer, superficial layer, and migration genes. Statistical comparisons between cortical neurons were performed using the Welch’s *t*-test (** $p < 0.01$).

(Figure 6D). Most of these top downregulated genes have roles in pluripotency (Madan et al., 2009; Hu et al., 2012; Vallot et al., 2013; Emani et al., 2015; Radine et al., 2020). There was a large overlap in the genes that were significantly upregulated and downregulated in cortical neuron vs hESC groups with high or low α Syn (Figure 7A). There was also a substantial overlap of gene ontology (GO) terms significantly upregulated and downregulated in the high or low α Syn comparisons (Figure 7B). The most significantly enriched KEGG pathway

in both high and low α Syn cortical neuron vs. hESC comparisons for upregulated genes was “axon guidance” (KEGG:04360). Five other top ten KEGG pathways linked with upregulated genes were the same for both the high α Syn and low α Syn cortical neuron vs hESC comparisons (Figure 7C). The “ribosome” term (KEGG:03,010) was the most significant downregulated pathway common to both comparisons. Interestingly, the Parkinson’s disease KEGG pathway was downregulated in high α Syn cortical neurons vs high α Syn hESCs, due to the

downregulation of several mitochondrial genes and cytochrome c oxidase genes, including *NDUFB9*, *MT-CO2*, *MT-CO3*, *MT-ATP6*, *MT-ATP8*, *MT-CYB*, *COX7C*, and *CYCS*. This may reflect a mitochondrial phenotype caused by elevated α Syn expression in cortical neurons. The data so far suggests that the process of cortical neuron differentiation induced a large number of significant gene expression changes, and that the level of α Syn in these cells does not impair this process.

The key group comparison was that of high α Syn cortical neurons vs. low α Syn cortical neurons. The volcano plot, in comparison to the cortical neurons vs. hESC plots, identified a total of 47 differentially expressed genes (**Figure 8A**; **Supplementary Table S1**). KEGG pathway analysis did not show any relevant neurogenesis-related pathways enriched in high α Syn vs low α Syn cortical neurons (**Figure 8B**). Selected gene expression analysis revealed that the pluripotency markers *POU5F1* (*OCT4*) and *NANOG* were significantly downregulated and neurogenesis markers, *ASCL1*, *MYT1L*, and *POU3F2*, were significantly upregulated in cortical neuron samples relative to hESC samples for both high α Syn and low α Syn groups (**Figure 8C**) (Nichols et al., 1998; Chambers et al., 2003; Vierbuchen et al., 2010). Analysis of genes implicated in axon guidance, including *MAP2*, *SLIT2*, *EPHBI* and *NCAM1* showed no difference in cortical neurons with low or high α Syn, except for *NTN1* ($p < 0.01$) (**Figure 8D**) (Kennedy et al., 1994; Izant and McIntosh, 1980; Borrell et al., 2012; Enriquez-Barreto et al., 2012). There were no significant differences in genes associated with synaptic development, in low α Syn and high α Syn cortical neurons, including *CADMI*, *NLGN1*, *SNAP25*, *SYP*, and *DLG4* (**Figure 8E**) (Wiedenmann and Franke, 1985; Oyler et al., 1989; Cho et al., 1992; Ichtchenko et al., 1995; Stagi et al., 2010). Other markers were explored to ascertain telencephalic development. *FOXP1* and *PAX6*, cortical progenitor markers, and *CTIP2* and *TLE4*, markers of deep cortical layers, showed no significant differences in low and high α Syn cortical neuron groups (**Figure 8F**) (Raciti et al., 2013). The superficial layer marker *SATB2*, was not significantly different between the low and high α Syn cortical neurons, which is in keeping with its involvement in the later stages of cortical development (Mariani et al., 2012). Expression of the neuronal migration marker, *DCX*, also presented no significant variation between low or high α Syn cortical neurons (**Figure 8F**) (Gleeson et al., 1999).

DISCUSSION

In this study, we generated and investigated 29 *SNCA* transgenic hESC lines and found no correlation between α Syn expression level and neural differentiation potential. We further addressed the hypothesis that increased α Syn expression does not impair cortical neuronal differentiation of human pluripotent stem cells. Across multiple clonal lines, multiple rounds of cortical neuron differentiation using two robust differentiation protocols and unbiased transcriptomic analysis, we show data to support this hypothesis. The level of *SNCA* expression, whether normal or increased, did not have a significant impact on the efficiency of cortical neuron differentiation. These findings resolve conflicting data in the field, and are highly relevant to studies utilising human pluripotent stem cells (hESCs or iPSCs) with differential α Syn

expression to model synucleinopathies, such as PD, DLB and multiple system atrophy (MSA).

As expected, significant differential gene expression was observed when cortical neurons were compared to undifferentiated hESCs for both high α Syn and low α Syn hESCs. Gene ontology (GO) and KEGG pathway analysis identified significant overlap in the group comparisons with high α Syn or low α Syn hESCs and their differentiated counterparts. Furthermore, most of the top differentially expressed genes were the same in these comparisons, indicating neurogenesis proceeded in a similar manner despite the level of α Syn expression. The gene expression differences that were unique to the high α Syn hESCs or to the low α Syn hESCs could be due to a number of factors, including clonal variation, experiment-to-experiment variation, or due to a phenotype of elevated α Syn expression, such as mitochondrial dysfunction. When high α Syn and low α Syn cortical neurons were directly compared to each other, only 47 genes were differentially expressed, and pathway analysis did not reveal anything relevant to cortical neuron induction or differentiation. Furthermore, read count analysis for well-characterised genes with known roles in neurogenesis and the development of cortical identity confirmed cortical differentiation occurred similarly between the high α Syn and low α Syn groups.

Research groups, including ourselves, have used hiPSCs derived from patients with *SNCA* multiplication mutations to model synucleinopathies (Byers et al., 2011; Devine et al., 2011; Flierl et al., 2014; Oliveira et al., 2015; Lin et al., 2016; Prots et al., 2018; Chen et al., 2019; Brazdis et al., 2020). We previously showed, using eight clonal hiPSC lines, harbouring the *SNCA* triplication mutation and six hiPSC lines from a non-affected first-degree relative, that the main sources of variation in dopaminergic differentiation efficiency were due to differences between clonal lines, reprogramming efficiency, and the process of neuronal differentiation itself (Devine et al., 2011). Whilst cellular reprogramming is a powerful method for generating human disease models, reprogramming can be incomplete and can introduce coding mutations or large chromosomal abnormalities that may lead to altered differentiation potential of different clones (Mayshar et al., 2010; Boulting et al., 2011; Hussein et al., 2011). Furthermore, in these patient-derived iPSCs the size of the multiplied region is variable and other adjacent genes such as *MMRN1* may be incorporated (Ross et al., 2008). Over-expression of other coding genes in these iPSC lines presents another potential confounding factor when investigating and interpreting neuronal differentiation potential. A number of these caveats apply to studies differentiating hiPSCs with a *SNCA* triplication into neuronal progenitors or dopaminergic neurons (Flierl et al., 2014; Oliveira et al., 2015). In particular, limited clonal lines were examined and siRNA knock-down of *SNCA* “rescue” of dopaminergic differentiation was partial (Oliveira et al., 2015).

To further highlight the point regarding clonal variation and conflicting reports in the literature, recent studies have shown, using hiPSCs from patients with a *SNCA* duplication mutation, that cortical neurons (Prots et al., 2018) and dopaminergic neurons can be efficiently and comparably generated using these hiPSC lines (Brazdis et al., 2020). Other independent

groups have also used hiPSCs with a SNCA triplication mutation to show functional dopaminergic neuron generation comparable to control hiPSC lines (Byers et al., 2011; Lin et al., 2016). We recently showed that reducing SNCA alleles in isogenic hESC lines also does not affect dopaminergic neuronal differentiation; wild type, SNCA^{+/-} and SNCA^{-/-} hESC lines showed no differences in differentiation into FOXA2:TH double-positive dopaminergic neurons (Chen et al., 2019). This study, and the triplication and duplication SNCA hiPSC studies (Devine et al., 2011; Brazdis et al., 2020) strongly predict that the isogenic collection of hESC lines generated here will have similar efficiencies of midbrain dopaminergic differentiation. In the studies reporting no impairment of dopaminergic neuron differentiation with a SNCA multiplications, PD-related phenotypes, including reduced synchronous firing on microelectrode recordings and increased susceptibility to oxidative stress were observed (Lin et al., 2016; Brazdis et al., 2020). While there may be α Syn-related phenotypic differences or inherent vulnerabilities due to elevated α Syn expression in mature neurons, this does not imply that the process of neuronal differentiation itself is impaired.

When α Syn was over-expressed using a lentiviral system in hESC-derived neuroectoderm impaired neuronal patterning and acute toxicity were reported (Schneider et al., 2007). Dopaminergic and GABAergic neuron populations were affected by α Syn over-expression, but this was only quantified in a single hESC line (H9), and the toxicity shown to be occurring could result in selective neuronal loss, making the interpretation of differentiation marker analysis challenging (Schneider et al., 2007). Similar caveats apply to work performed using AF22 neural stem cells with doxycycline-inducible α Syn expression. Exposure to doxycycline may cause pleiotropic effects, and α Syn expression was not sustained throughout differentiation (Zasso et al., 2018).

It is important to consider that neuronal differentiation protocols often require optimisation for each clonal line and small variations in ligand concentrations, specifically CHIR99021 in the midbrain dopaminergic differentiation protocol, can impair neurogenesis. For example, in the study by Oliveira et al., 2015 the dopaminergic differentiation protocol yielded less than eight percent TH-positive neurons in the control lines. Whilst our study does not yield insight into the impact of elevated α Syn expression on dopaminergic differentiation, a subject for future studies, the cortical neuron protocols used were robust with the vast majority of cells producing cortical neurons.

The next steps for these cell lines are to investigate differential susceptibility to know triggers of synucleinopathy, such as environmental risk factors that affect autophagy and lysosomal biology, as well as compounds that inhibit mitochondrial function. We have previously shown that reduction of SNCA alleles in hESC-derived neurons protects against synucleinopathy (Chen et al., 2019), and it is predicted that increasing α Syn expression will make neurons more susceptible to conditions that promote Parkinson's and other Lewy body conditions.

In summary, this work has produced an allelic series of isogenic clonal hESC lines with differing levels of α Syn expression, and we have demonstrated that elevated α Syn expression does not impair cortical neurogenesis. This

supports the validity of using human pluripotent stem cells, such as iPSCs with SNCA multiplications and transgenic hESC lines, to model synucleinopathies. Since this collection of cell lines are isogenic and the only genetic variable is the level of α Syn expression, their utility for disease modelling and research into novel therapeutics is very significant. However, close matching of the differentiation stage and maturity of neurons from the different clones is still required for accurate interpretation of results. The practical application of these cell lines is most promising for investigating treatments that are targeted against α Syn itself or downstream effects of its over-expression.

METHODS

Generation of Transgenic hESC Lines by Nucleofection

Shef4 hESCs were provided Prof D Hay (University of Edinburgh) following MRC Steering Committee approval (SCSC11-60). The plasmid consisting of wild-type human SNCA (pcDNA3.1) was provided by Prof J Hardy (UCL). The PGK-Puro-pCAGS and FCT-IRES-Venus-pBS plasmids were provided by William Hamilton (University of Edinburgh).

IRES-Venus and human SNCA fragments were amplified by PCR (MJ Research, PTC-200 Peltier Thermal Cycler) and purified using DNA Clean and ConcentratorTM-5 Kit (Zymo Research, D4003) to provide 20 μ l of eluted DNA. 1X BSA, 1X digestion buffer and 50 U digestion enzyme (all New England Biolabs[®]) were used to digest the plasmid DNA, which was then purified from agarose gel using ZymocleanTM Gel DNA Recovery Kit (Zymo Research, D4001) per manufacturer's protocol. Purified and digested DNA was ligated to PCR products at 16°C overnight in a final volume of 20 μ l using 2 U of T4 DNA ligase and 1x ligation buffer (Roche, 10481220001). Plasmid DNA was transformed into TOP10 chemically competent cells (Invitrogen, c4040-10) by the heat-shock method. Plasmid DNA was extracted using QIAprep[®]-Spin Miniprep kit (Qiagen, 27,104) or Maxiprep (Qiagen, 12,662) systems per manufacturer's protocol, then desalted using Millipore centrifugal filter units (Millipore, UFC503024 24PK). The final pCAG-SNCA-IRES-Venus construct contained human SNCA, internal ribosome entry site (IRES) and Venus expression cassette under the constitutive pCAG promoter, as well as a puromycin resistance gene (Puro^r) driven by the PGK promoter. The control construct, pCAG-IRES-Venus, contained the same elements except for the SNCA gene (**Figure 1A**).

The Neon Transfection System (Invitrogen, MPK5000) was used for nucleofection of both constructs into Shef4 hESCs as per the manufacturer's protocol. 1 μ g/ml puromycin was added to the culture media for at least two weeks to isolate clones and colonies, manually picked for expansion and cryopreservation. Clones were screened for SNCA over-expression by qRT-PCR.

Quantitative RT-PCR

The MasterPureTM Complete DNA and RNA Purification kit (Epicentre, MC85200) or the RNeasy kit (Qiagen, 74,104) was used for RNA extraction. Genomic DNA was removed using DNase I (Promega, M6101). cDNA was synthesised from 500 ng

total RNA using M-MLV reverse transcriptase (RT, ThermoFisher Scientific, 28025013) or Superscript IV reverse transcriptase (Invitrogen, 18090010). qRT-PCR was performed using a LightCycler™ 480 (Roche) with the following parameters: (95°C for 10 min), [(95°C for 10 s) + (60°C for 20 s)] over 45 cycles. Intron-spanning primers were designed using the universal Probe Library (UPL) Assay design centre (Roche). Primer sequences and UPL probes were total *SNCA* F-tgggcaagaatgaagaaggagc, R-gtggtagcgggtgtgacagc Probe 68; transgenic *SNCA* F-cgacctcagctggacct, R-tgacaatgacatccactttgc Probe 163; *NCAM* F-gcgttgagagtgcaaatc, R-gggagaaccaggagatgtcttt Probe 51; *MAPT* F-accacagccacttctct, R-cagccatctggttcaagt Probe 55; *PAX6* F-tca ccatggcaataaacctg, R-cagatgcaggagatgagg Probe 20; *TBR1* F-aggaagaagaatggcctaaaa, R-ggatgcatatagaccggatttc Probe 22; TATA-box binding protein (*TBP*) F-ataggattccgggagctcat, R-gaacatcagatgagaacaaca Probe 87. Each 10 μ l reaction was performed in triplicate and results normalised to *TBP* expression.

MTS Assay

Cell proliferation was assessed using a colorimetric assay, CellTiter96® AQueous One Solution Cell Proliferation Assay or MTS assay. In this test, MTS tetrazolium is bio-reduced in viable cells (Supplementary Figure S1).

hESC and hiPSC Culture

Shf4-derived transgenic hESC lines, and AST18 and NAS2 hiPSCs were expanded in culture on either Matrigel-coated 6-well plates (BD, 356234) in mTeSR1 medium (Stemcell™ Technologies, 05850) or on Laminin-521 (BioLamina, LN521) coated 6-well plates in StemMACS iPS-Brew XF, human (Miltenyi Biotec, 130-107-086). 1 μ g/ml puromycin (Sigma, P8833) was used in the culture media of Tg hESCs to maintain the expression of the transgenes.

Cortical Neuronal Differentiation

Two cortical neuron differentiation protocols were used, referred to as CD protocol 1 and CD protocol 2, respectively. CD protocol 1 was adapted from the cortical neuron differentiation protocol published by Chambers et al., 2009 and CD protocol 2 was adapted from the published protocol by Shi et al., 2012. Both protocols use dual SMAD inhibition to induce cortical neuron differentiation. In CD protocol 1, neural differentiation was started by changing the culture media to neural induction media (NIM) which included 10 μ M SB431542 (Tocris, 616461) and 100 nM LDN-193189 (Stemgent, 04-0019). NIM for this protocol was prepared by mixing 1:1 DMEM/F12 (Gibco, 20331-020) and Neurobasal medium (Gibco, 21103-049), supplemented with 1 ml N2 and 2 ml B27 with retinoic acid (Gibco, 17504-044). NIM was also supplemented with 2 mM L-glutamine, 0.1 mM β -mercaptoethanol (BDH, 44143-31), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, 15140-122) and 100 μ M non-essential amino acids (Gibco, 1140-035). Cells were lifted using dispase at day 12, dissociated into clumps and plated on 10 μ g/ml Laminin-111 (Sigma L2020-1 MG) and 15 μ g/ml poly-L-ornithine-coated plates (Sigma, P4957). 100 nM LDN and 20 μ g/ml FGF2 were included in the NIM, and SB431542 removed from day 12. Following 7–10 days of progenitor

colony expansion, cells were lifted using accutase and plated onto Laminin-111/poly-L-ornithine-coated plates in NIM supplemented with 10 ng/ml BDNF (Peprotech, 450-02) and 10 ng/ml GDNF (Peprotech, 450-10). Half media changes were performed every 3 days during the neuronal maturation phase up to day 83.

In CD protocol 2 hESCs at 80%–90% confluency in 6-well plates were lifted with 1 ml/well UltraPure 0.5 M EDTA (Invitrogen, 15575038), counted, and transferred onto 5 μ g/ml Laminin-111 coated (Biolamina, LN111-04) 24-well plates (Corning, 3527), at an initial plating density of 80,000 cells/cm². 600 μ l/well neural induction media (NIM) was used until day 4 of differentiation. NIM was composed of 50% DMEM/F12 (ThermoFisher Scientific, 21331020) and 50% Neurobasal Media (ThermoFisher Scientific, 21103049), B27 supplement with Retinoic Acid (ThermoFisher Scientific, 17504044), N2 supplement (ThermoFisher Scientific, 17502048) and 2 mM L-Glutamine (ThermoFisher Scientific, 25030123). From day 4 onwards, 50% NIM, 25% DMEM/F12 and 25% Neurobasal Media with 2 mM L-glutamine. For the first 12 days of differentiation, 10 μ M SB431542 (Tocris, 616461) and 100 nM LDN-193189 (Miltenyi Biotec, 130-103-925) were added to the NIM and this media was replaced every two days. Cells were lifted at day 12 and day 17 with Collagenase Type IV (Life Technologies, 17104019) diluted in HBSS (ThermoFisher Scientific, 14025). Cells were lifted and passaged as clumps in a ratio of 1:1.5 and 1:2 at day 12 and 17, respectively. 10 μ M Y27632 (Tocris) was used in the media for each cell lift. At day 25, differentiated cells were lifted with Accutase (Sigma, A6964) and cell pellets frozen using a dry ice and ethanol bath for RNA isolation.

Transcriptomic Analysis

Total RNA was isolated with RNeasy kit (QIAGEN, 74104). RNA integrity (RIN \geq 7) was confirmed using TapeStation 4200 (Agilent). The median RIN score was 9.2. Samples were collected at day 0 and day 25, across three sets of cortical neuron differentiations (Figure 5A). The samples were processed by Qiagen Genomic Services using their QIAseq UPX 3' Transcriptome kit and libraries were sequenced on a NextSeq500 instrument. The total number of polyadenylated 3' transcript reads and the mean number of reads per unique molecular identifier were counted. The raw transcript counts were analysed using DESeq2 (Love et al., 2014) differential expression analysis in R studio. Pairwise analysis was used to compare hESC vs. cortical neurons with high or low α Syn. A padj cut off value of 0.05 and a log₂ fold-change cut off value of 1.2, were used. KEGG pathway analysis was performed using the gGOST function in the CRAN gprofiler2 package (Raudvere et al., 2019). RNA-seq data has been deposited on the Gene Expression Omnibus (GEO Accession number: GSE195877).

Immunocytochemistry

Immunostaining was performed on cells cultured on 13-mm glass coverslips or in Ibidi 8-well plates. Spent medium was removed and cells fixed with 4% PFA for 15 min. Following 3 PBS (ThermoFisher Scientific) washes, the cells were permeabilised and blocked with 2% goat (or 2% donkey) serum (Sigma) in 0.1% Triton-X-100 (Fisher) in PBS for 45 min prior to overnight incubation at 4°C with primary antibodies. The primary antibodies used were β -III tubulin (1:1,000,

mouse IgG2b, Sigma T8660), CTIP2 (1:500, rat IgG2a, Abcam ab18465), PAX6 (1:40, mouse IgG1, DSHB ab528427), TBR1 (1:200, rabbit IgG, Abcam ab31940), OTX1/2 (1:1,000, rabbit IgG, Abcam ab21990), vimentin (2 μ g/ml final, mouse IgM, DSHB 40E-C), and total α Syn (1:1,000, mouse IgG1, BD 610787). Secondary antibodies were applied at 1:1,000 dilution at room temperature for 1 h in the dark. These were goat anti-mouse IgG1 Alexa Fluor 488 (ThermoFisher Scientific, A21121), goat anti-mouse IgG2a Alexa Fluor 488 (ThermoFisher Scientific, A21131), donkey anti-rat IgG Alexa Fluor 488 (ThermoFisher Scientific, A21208), goat anti-mouse IgG1 Alexa Fluor 555 (ThermoFisher Scientific, A21127), goat anti-rabbit IgG Alexa Fluor 555 (ThermoFisher Scientific, A21428), donkey anti-rabbit IgG Alexa Fluor 555 (ThermoFisher Scientific, A31572), goat anti-mouse IgG2b Alexa Fluor 647 (ThermoFisher Scientific, A21242), goat anti-mouse IgM Alexa Fluor 568 (ThermoFisher Scientific, A21043), and donkey anti-mouse IgG Alexa Fluor 647 (Abcam, ab150107). Following a further three PBS washes, slides were mounted using Fluorsave (Merck, 345789). 0.1 μ g/ml 4',6-Diamidino-2-Phenylindole (DAPI, Life Technologies) was used to stain nuclei.

Image Capture, Processing and Quantification

The Eclipse Ti (Nikon) and/or the Axio Observer (Zeiss) microscopes were used to acquire the images presented. Huygens Software (Scientific Volume Imaging) was used for deconvolution of Z stack images (at least 10 images/stack and maximum intensity pixel projection used). Fiji software was used for image analysis and quantification, with identical brightness and contrast values used for each image channel and for each experiment. Macro scripts were used to split the image channels, threshold and binarize for quantification.

Western Blotting

RIPA Lysis Buffer (Santa Cruz, sc-24948) was used to lyse cell pellets and protein concentration determined using the Micro BCA Protein Assay kit (ThermoFisher Scientific, 232350). 10 μ g protein, per sample, was mixed and incubated with 5 μ l NuPAGE™ LDS Sample Buffer (ThermoFisher Scientific, NP0007) and 2 μ l 1 M DTT (ThermoFisher Scientific, NP0004) prior to loading on a NuPAGE™ 4%–12% Bis-Tris Gradient Gel (ThermoFisher Scientific, NP0322BOX). SeeBlue™ Plus2 Pre-stained Protein Standard (5 μ l, ThermoFisher Scientific, LC5925) was used as a standard. Following electrophoresis, protein was transferred onto a 0.45 μ m nitrocellulose (Amersham Protran Premium, 10600096) or a PVDF membrane (GE Healthcare Amersham Hybond ECL, RPN68D). 0.4% PFA was used to fix the protein on the membrane. One minute of methanol (Fisher Scientific, M/3900/17) immersion was done, in addition, if a PVDF membrane was used. Membranes were immersed in blotting-grade blocker (BioRad, 1706404) in 0.1% TBS-Tween for an hour at room temperature and then the primary antibody, mouse anti- α Syn (1:1,000, BD 610787) was added overnight at 4°C. Following three washes in 0.1% TBS-Tween, 1:2,000 HRP-conjugated anti-mouse IgG (Promega) secondary antibody was applied for two hours at room temperature. Pierce™ ECL Western

Blotting Substrate (ThermoFisher Scientific, 32109) was then added to the membrane for image capture using the LI-COR Odyssey imaging system (Biosciences). Antibodies were stripped with the Restore™ PLUS Western Blot Stripping Buffer (ThermoFisher Scientific, 46430). The blocking step was repeated and secondary antibody, HRP-conjugated anti- β -Actin antibody (1:1,000, Abcam) applied. The Pierce™ ECL Western Blotting Substrate was again used prior to imaging the membrane.

Statistics

Statistical tests were performed using SPSS v23 and include the Student's *t*-test or *t*-test with Welch's correction, linear regression analysis and the Mann-Whitney *U* test for non-parametric data. Each Figure legend details which test was used for each statistical comparison. Significance level cut off was $p < 0.05$.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

TK conceived the project. TK, AN, RY designed the experiments. AN and RY performed the experiments and analysed the data. RB performed bioinformatic analysis. AN and TK wrote the manuscript, and all authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.898560/full#supplementary-material>

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