



RESEARCH NOTE

Discovery of functional non-coding conserved regions in the α -synuclein gene locus [v1; ref status: approved 1, approved with reservations 1, <http://f1000r.es/2pt>]

Lori Sterling¹, Michael Walter², Dennis Ting¹, Birgitt Schüle¹

¹Parkinson's Institute and Clinical Center, Sunnyvale, CA 94085, USA

²Institute of Human Genetics, Eberhard-Karls-University Tübingen, Tübingen, 72076, Germany

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Abstract

Several single nucleotide polymorphisms (SNPs) and the Rep-1 microsatellite marker of the α -synuclein (*SNCA*) gene have consistently been shown to be associated with Parkinson's disease, but the functional relevance is unclear. Based on these findings we hypothesized that conserved cis-regulatory elements in the *SNCA* genomic region regulate expression of *SNCA*, and that SNPs in these regions could be functionally modulating the expression of *SNCA*, thus contributing to neuronal demise and predisposing to Parkinson's disease.

In a pair-wise comparison of a 206kb genomic region encompassing the *SNCA* gene, we revealed 32 evolutionary conserved DNA sequences between human and mouse. All elements were cloned into reporter vectors and assessed for expression modulation in dual luciferase reporter assays. We found that 11 out of 32 elements exhibited either an enhancement or reduction of the expression of the reporter gene. Three elements upstream of the *SNCA* gene displayed an approximately 1.5 fold ($p < 0.009$) increase in expression. Of the intronic regions, three showed a 1.5 fold increase and two others indicated a 2 and 2.5 fold increase in expression ($p < 0.002$). Two elements downstream of the *SNCA* gene showed 1.5 fold and 2.5 fold increase ($p < 0.0009$). One element downstream of *SNCA* had a reduced expression of the reporter gene of 0.35 fold ($p < 0.0009$) of normal activity.

Our results demonstrate that the *SNCA* gene contains cis-regulatory regions that might regulate the transcription and expression of *SNCA*. Further studies in disease-relevant tissue types will be important to understand the functional impact of regulatory regions and specific Parkinson's disease-associated SNPs and its function in the disease process.

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report



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- 1 **Ornit Chiba-Falek**, Duke University USA,
Lidia Tagliaferro, Duke University USA
- 2 **Jinglan Liu**, St. Christopher's Hospital for
Children USA

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Corresponding author: Birgitt Schüle (bschuele@thepi.org)

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Introduction

An emerging hypothesis is gaining increasing interest and is based on the concept that subtle overexpression of α -synuclein (α -syn) over many decades can either predispose or even cause the neurodegenerative changes that characterize Parkinson's disease (PD). Neurons subjected to higher, non-physiological levels of α -syn might be more likely to be damaged by oligomerization or aggregation of this protein, eventually leading to the formation of α -synuclein-based neuropathological features of the disease¹.

It is now well established that both point mutations and large genomic duplications of the α -syn (*SNCA*) gene can cause an autosomal-dominant form of PD²⁻¹⁰. Furthermore, several association studies investigating genetic variants in the *SNCA* gene have found an increased risk for PD¹¹⁻¹⁹. The finding that both qualitative and quantitative alterations in the *SNCA* gene are associated with the development of a parkinsonian phenotype indicates that amino acid substitutions as well as overexpression of wild-type α -syn are capable of triggering a clinicopathological process that is very similar to sporadic PD. Nevertheless, the precise mechanisms leading to α -syn-related pathology in sporadic PD in the absence of any α -syn mutations remain elusive.

The best characterized polymorphism in the *SNCA* gene is the Rep-1 mixed dinucleotide repeat which has been shown to act as a modulator of *SNCA* transcription¹⁴⁻¹⁶. The DNA binding protein and transcriptional regulator PARP-1 showed specific binding to *SNCA*-Rep1. These data were confirmed by a transgenic mouse model and demonstrated regulatory translational activity²⁰.

Functionally, *SNCA* expression levels in postmortem brains suggest that the Rep-1 allele and SNPs in the 3' region of the *SNCA* gene have a significant effect on *SNCA* mRNA levels in the substantia nigra and the temporal cortex²¹.

The promoter region of the *SNCA* gene has been recently examined in more detail in cancer cell lines and also in rat cortical neurons. Regulatory regions in intron 1 and the 5' region of exon 1 have been shown to exhibit transcriptional activation²²⁻²⁴ as well as the NACP-Rep-1 region upstream of the *SNCA* gene^{14-16,20,25}. Several transcription factors have been identified such as PARP-1¹⁶, GATA²⁶, ZIPRO1, and ZNF219²² to have an effect on regulating the *SNCA* promoter region.

There is mounting evidence that *SNCA* expression levels could be crucial for maintenance and survival of neurons and its misregulation could play a key role in the development of PD. Thus, the importance of thoroughly investigating the *SNCA* gene to fully understand its cis- and trans-acting elements and factors and for the functional interpretation of the PD-disease associated risk alleles is becoming increasingly clear.

The goal of this study was to investigate transcriptional regulation of the *SNCA* region using a complementary approach, under the hypothesis that conserved non-coding regions of the *SNCA* gene are comprised of transcriptional enhancers or silencers and thus modulate gene expression. This would mean that single nucleotide polymorphisms (SNPs) in these regions could influence the transcriptional pattern of the *SNCA* gene²⁷.

Materials and methods

Comparative genomics

Using comparative genomics, we searched for highly conserved non-coding sequences between human and mouse and identified 37 evolutionary conserved non-coding genomic regions (ncECRs) within the *SNCA* gene that are conserved between human and mouse.

We utilized two complementary browsers (Vista browser (<http://pipeline.lbl.gov/cgi-bin/gateway2>) and ECR browser (<http://ecr-browser.dcode.org/>) to generate a conservation profile by aligning the human *SNCA* gene with its mouse counterpart in a pair-wise fashion. We applied established selection parameters for our search with >100bp in length and >75% identity^{28,29}. In addition to the 111.4kb *SNCA* gene region, we included a 44.5kb upstream and a 50kb downstream intergenic region to also capture surrounding regulatory elements.

We identified 37 ncECRs in the *SNCA* genomic region of 206kb on chromosome 4q21 (Chr.4: 90,961056-91,167082, UCSC Genome Browser on Human Mar. 2006 Assembly) by pair-wise comparison between human and mouse (Figure 1). Ten of these DNA sequences were located downstream of the *SNCA* gene, 17 were intronic between exon 4 and 5, which is 92kb in length, and five were upstream of the *SNCA* gene (Figure 1). None of the selected sequences overlapped with known expressed sequence tags (ESTs) or had an open reading frame of more than 20 amino acids in length, suggesting that these ncECRs are non-coding.

Cloning and luciferase assays

To test, if the ncECRs exhibit enhancer or silencer activity, we cloned all identified regions in specific reporter vectors and measured their luciferase activity after transfection into neuroblastoma cells. For our studies, we used the pGL3 luciferase reporter vectors (Promega, Cat. No. E1751, E1741, E1771, E1761) and the human neuroblastoma cell line SK-N-SH. NcECRs identified through the comparative analysis (Supplementary Table 1) were cloned upstream of a SV-40 promoter in the pGL3 promoter construct, transfected in SK-N-SH cells and assayed with the Dual-Luciferase® Reporter Assay System (Promega, Cat. No. E1910).

Some of these regions were combined in one vector because of their close proximity to each other. Primers with specific restriction sites (KpnI, BglII or XhoI from New England Biolabs Inc.) were designed to amplify the conserved elements, and PCR products with specific restriction sites were directly cloned into the pGL3 promoter vector to ensure correct orientation of the genomic elements (Supplementary Table 1). All constructs were sequenced to ensure that no point mutations were introduced through the amplification and/or cloning process.

For transfection experiments, we used a 96-well format (Nunc, Cat. No. 167008). Cells were plated one day before transfection at a density of 3000–5000 cell/well to reach 90–95% confluency at the time of transfection, luciferase assays were performed 24hrs after transfection. SK-N-SH cells were maintained in Hyclone DMEM media (High Glucose, Fisher Scientific, Cat. No. SH30081.02) with 10% Hyclone fetal bovine serum (Fisher Scientific, Cat. No. SH30910.03) in 1× glutamine (Life Technologies, Cat. No. 25030-081) and 1× penicillin/streptomycin (Life Technologies, Cat. No. 15140-122).

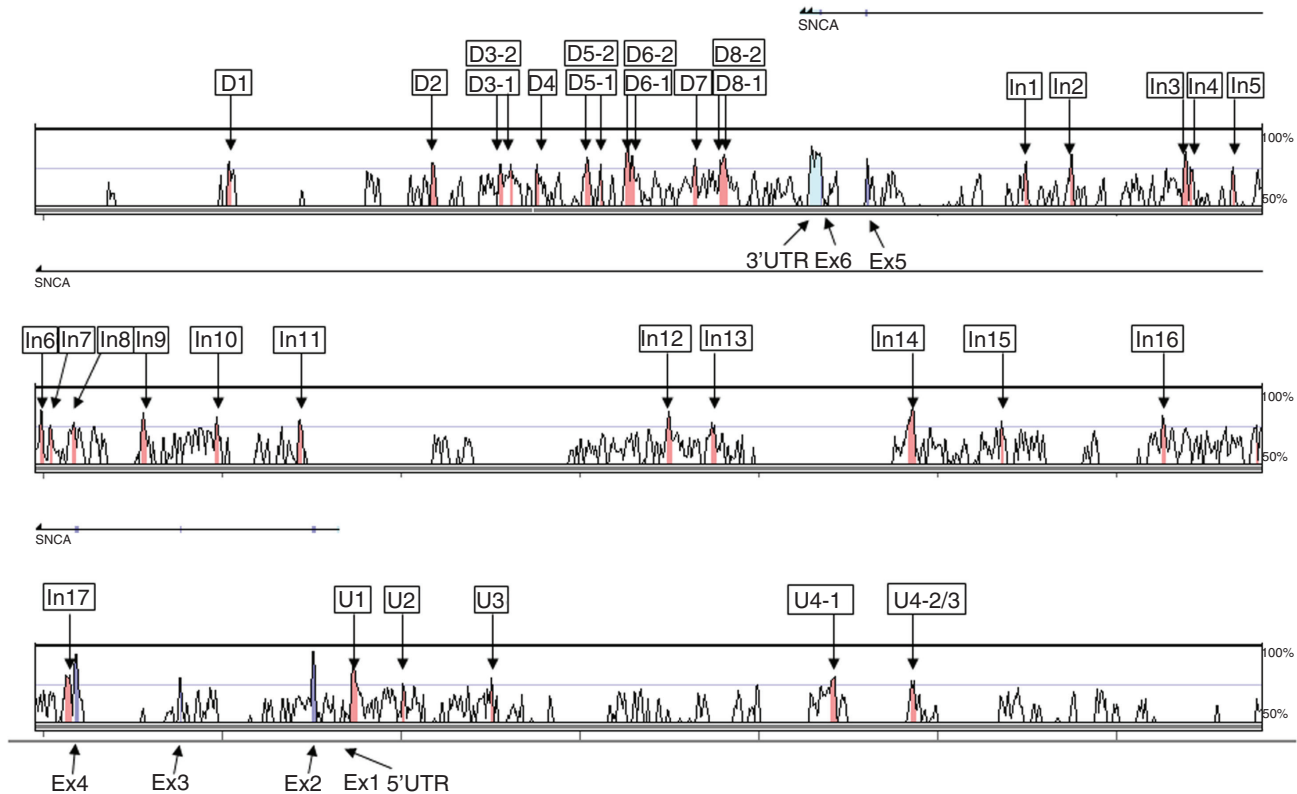


Figure 1. Vista plot from the *SNCA* region on chromosome 4q21. Panel shows human-mouse pair-wise comparison of Human genome May 2004 and Mouse Sept. 2005. Pink marked peaks represent ncECRs, turquoise marked peak represent the untranslated region (UTR) of *SNCA*, blue marked peaks represent exons. D1-D10 are conserved regions downstream of *SNCA*. In1-In17 are intragenic conserved regions, and U1-U4-2/3 are upstream of *SNCA*. The black arrow on top shows the transcription orientation.

For SK-N-SH cells, we used 1:2 ratio of nucleic acid to transfection reagent (Lipofectamine® 2000 Transfection Reagent, Life Technologies, Cat No. 11668-019). For the luciferase assay, we used the Dual-Luciferase® Reporter (DLR™) Assay System (Promega, Cat. No. E1910) according to the manufacturer's instructions in 96-well white plates, flat bottom (E&K Scientific, Cat. No. EK-25075). In this assay, activities of firefly and *Renilla* luciferases were measured sequentially in one sample. All assays were performed in quadruplicate and each experiment was repeated three times. Altogether, 12 data points were ascertained for each conserved region/construct.

Bioinformatic search for transcription factor binding sites (TFBS) with MatInspector (Genomatix)

To estimate the number of potential TFBSs and the number of interacting transcription factors (TFs) that could represent potential candidate proteins for our positive ncECRs, we used MatInspector in an *in silico* approach. We chose two elements for this bioinformatic analysis with MatInspector. The MatInspector software utilizes a large library of matrices for TFBSs to locate matching DNA sequences. The program assigns quality rating to matches and allows quality-based filtering and selection of matches. MatInspector can group similar or functionally related TFBSs into matrix families³⁰.

In addition to the original human-mouse comparison, we added the sequences for dog and cow for comparisons. Only the TFBSs were

considered that were present in all four species, in the same orientation, and similar distance to each other. We ran two analyses with 10 and 15 nucleotides distance, respectively. We accepted only models in which at least four TFs can bind in a concerted way. Each TFBS can potentially bind several TFs.

We also computationally tested all possible TFs for interactions with the *SNCA* promoter region, which were retrieved from the proprietary EIDorado database (Genomatix, Munich, Germany). In this database, promoters are defined and ranked by transcription start sites, corresponding known mRNA or EST sequences and by orthologous conservation.

Results

Functional non-coding conserved elements within the *SNCA* genomic locus

Overall, 12 of 37 conserved non-coding elements exhibited either an increase or reduction of the expression of the luciferase reporter gene (Figure 2 and Dataset 1). Three elements upstream of the *SNCA* gene (U3, U4-1, and U4-3) displayed a significant approximately 1.5 fold ($p < 0.009$) increase in expression (Figure 2A). Of the intronic regions, three showed a 1.5 fold increase (I2, I6, I8) and two others showed a 2 and 2.5 fold increase in expression ($p < 0.002$), I5 and I12, respectively (Figure 2B). Two elements downstream of the *SNCA* gene showed approximately 2 fold (D1 and D2) and 2.5 fold (D3) increase ($p < 0.0009$) (Figure 2C). One

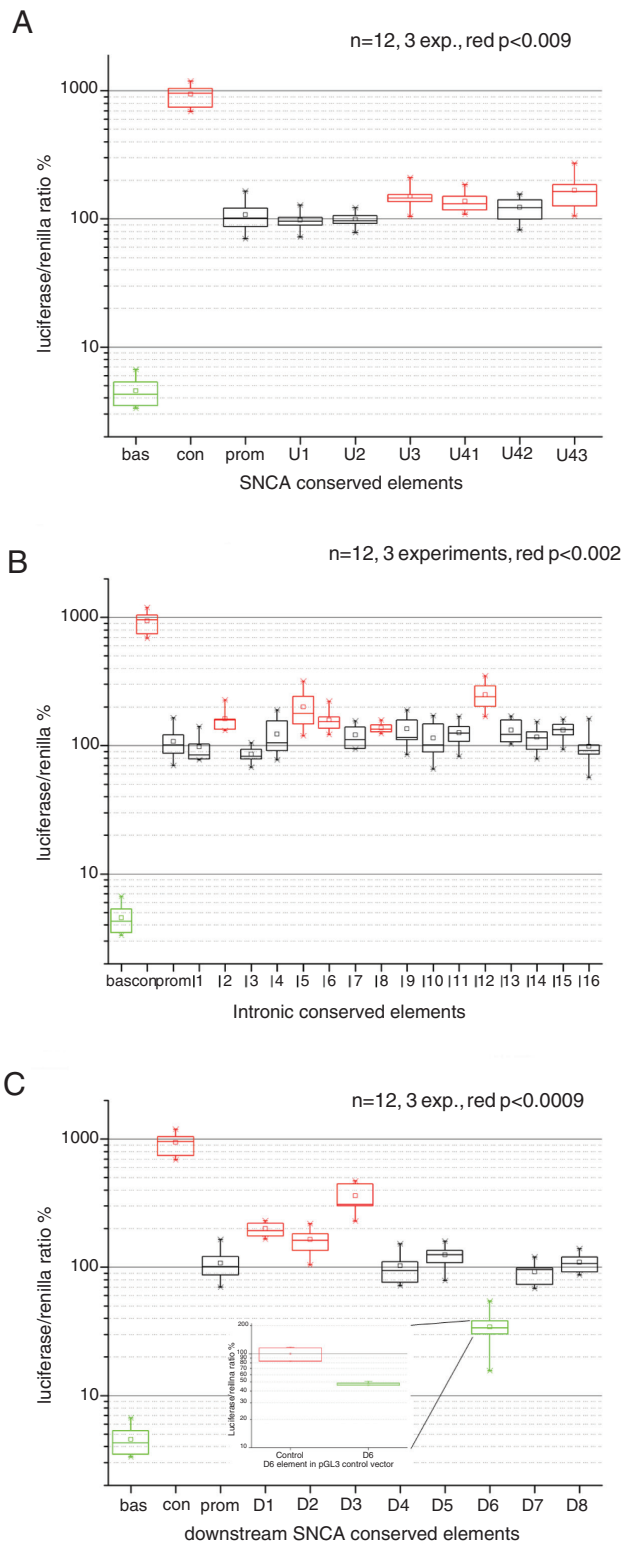


Figure 2. Non-coding conserved elements within the *SNCA* genomic locus show changes in luciferase assays. Panels **A–C** show the luciferase assay results of ncECRs upstream (**A**), intragenic (**B**), and downstream (**C**) of the *SNCA* gene. The X-axis shows the ncECRs, the Y-axis shows the ratio of luciferase and renilla expression as percentage. Bas=pGL3 basic, Con=pGL3 control, prom=pGL3 promoter construct. All red or green box plot elements represent ncECRs that modulate expression significantly. The box plots show the median (horizontal line within box), the 25 and 75% tiles (horizontal borders of box), and the whiskers show the minimal and maximal values. Panel **C**, insert: Luciferase assay results of D6 element cloned into the pGL3 control vector construct.

element D6 downstream of *SNCA* had a reduced expression of the reporter gene of 0.35 fold ($p < 0.0009$) of normal activity (Figure 2C, green) that was also confirmed after cloning the D6 element in a pGL3 control vector (Figure 2C, insert). The pGL3 control vector contains the SV-40 promoter and a SV-40 enhancer element. The D6 element reduced the expression of the pGL3 control construct by ~50%, confirming that this element represents a repressor. Between 4 and 12 replicates were performed per ncECR.

These data provide experimental evidence that a significant proportion of the ncECRs show a regulatory function in the luciferase reporter assay.

In silico analysis reveals potential binding of midbrain transcription factors to regulatory conserved regions

We performed MatInspector (Genomatix) analysis³⁰ on two elements (I12 and D6) with the highest fold change in the luciferase assay. In addition to the original human-mouse comparison to identify the ncECRs, we added the sequences from dog and cow. Only TFBSs that were present in all four species, in the same orientation, and similar distance to each other were considered. We ran two analyses with 10 and 15 nucleotides distance, respectively. We accepted only models in which at least four TFs can bind in a concerted way. Each

TFBS can potentially bind several TFs. Interestingly, using this more restricted model, five factors showed an interaction with the *SNCA* promoter as well as with the ncECRs (Figure 3A). These factors were the Paired-like homeodomain transcription factor 3 (PITX3), the Homolog of *Drosophila* orthodenticle 2 (OTX2), the Nuclear receptor subfamily 3, group c, member 1 (NR3C1) or glucocorticoid receptor (GCCR), the Androgen receptor (AR), and the general transcription initiation factor TATA box-binding protein (TBP).

It is intriguing to note that by searching for TFs that bind to both the promoter and the functional ncECR, several DNA-binding proteins were found that are linked to dopaminergic regulation and susceptibility for nigrostriatal impairment. Two of these TFs (PITX3 and OTX2) implicated in determination of a dopaminergic phenotype in the substantia nigra emerged from this preliminary search^{31,32}. PITX3 has shown to be regulated in a negative feedback circuit through the microRNA mi-133b to fine-tune maintenance of dopaminergic neurons³³. In an association study, a SNP in the PITX3 promoter was reported to be associated with PD and might dysregulate expression of PITX3³⁴ suggesting that transcription factors play a critical role not only in the development and differentiation of dopaminergic neurons, but also for cell maintenance and survival of dopaminergic neurons.

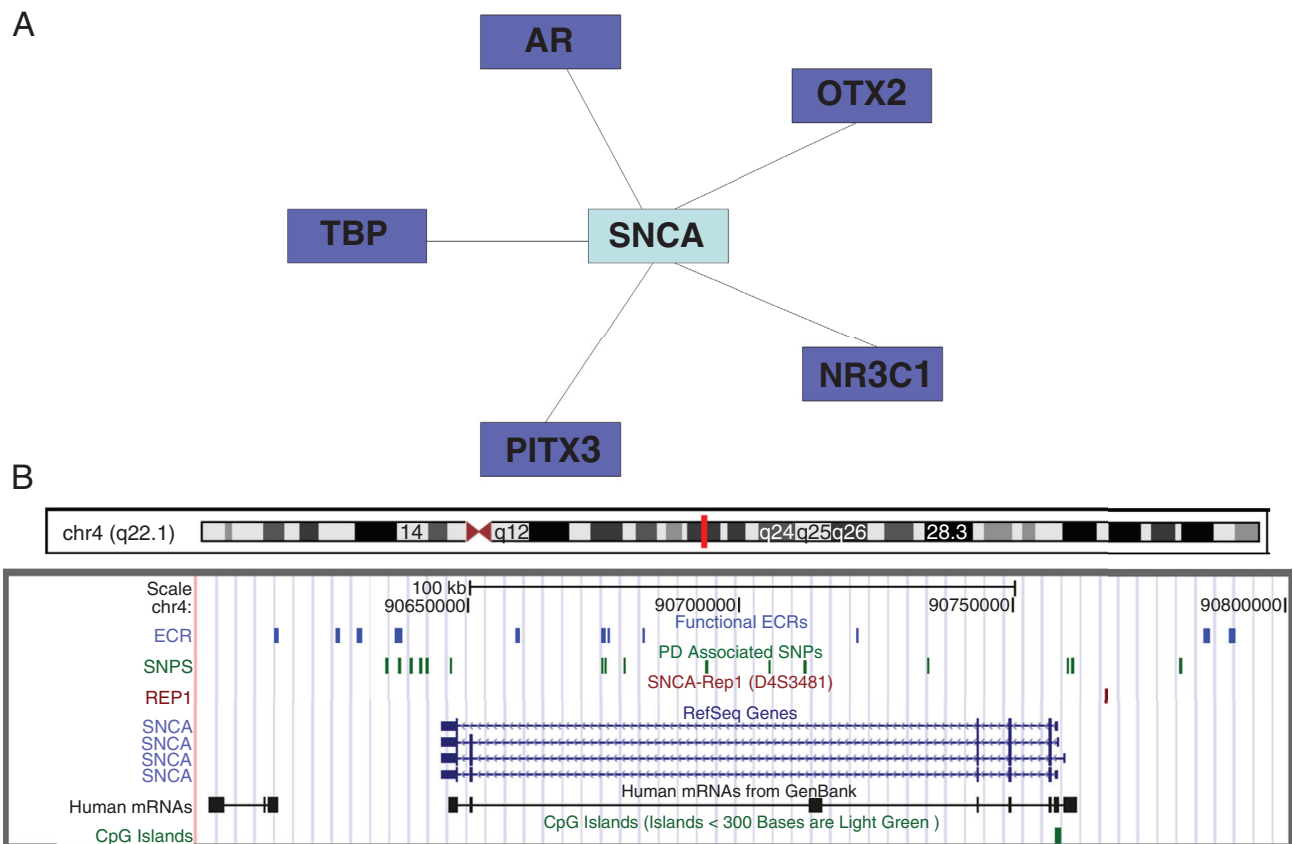


Figure 3. In silico analysis reveals midbrain transcription factors binding to two ncECRs. **A.** Scheme of *SNCA* interaction with TFs that also potentially bind to two ncECRs within the *SNCA* gene. **B.** UCSC Genome browser custom track of PD associated SNPs (based on PD Gene metaanalysis), Rep1 allele and functional ECR regions on chromosome 4 (Human Genome Assembly Feb. 2009, GRCh37/hg19).

GCCR and AR belong to a class of nuclear receptors called activated class I steroid receptors. GCCR is a cytosolic ligand-activated transcription factor that regulates the expression of glucocorticoid-responsive genes. GCCR shows strong anti-inflammatory and immunosuppressive effects. Interestingly, impaired GCCR expression in a mouse model shows a dramatic increase in the vulnerability of the nigrostriatal dopaminergic neurons to a toxic insult of MPTP³⁵.

Taken together, this preliminary *in silico* screen resulted in very intriguing new candidates that might directly regulate *SNCA* expression and could play a role in the pathological processes that underlie PD.

Dataset 1. Combined normalized raw datasets of Luciferase assays on *SNCA* conserved elements

<http://dx.doi.org/10.5256/f1000research.3281.d37452>

Data are ratios of luminometer readings for firefly luciferase and renilla luciferase. Ratios were normalized to Prom. Each non-coding element is labeled and data are presented under each element. Elements are organized according to [Figure 2A–C](#).

Discussion

A major focus in PD research has been on post-translational modification of α -syn. The alterations seen in PD that were linked to disease pathogenesis were nitrated α -syn and α -syn phosphorylated at serine 129 identified in Lewy bodies and Lewy neurites^{36,37}, however, the gene transcription as a control point and its regulation in particular cell types or upon cellular signals has only been touched fairly recently in PD-relevant genes.

Our results show that potential regulatory regions are not restricted to the promoter of the *SNCA* gene as discussed in the introduction, but are likely to be located also in other intronic and intergenic regions ([Figure 3B](#)). Comparing our results to similar screens, where conserved regions range from 8–45 elements^{38,41}, we found a similar number of functional elements in our screen that show a high evolutionary conservation.

Not only the promoter region of a gene drives the transcription/expression of a gene. Also other cis-acting genomic regions within a certain gene, up to several hundred kb away, can serve as enhancers, silencers, or modifiers to ensure the accurate temporal and spatial expression of a gene by recruiting transcription activating or silencing factors that bind to them³⁸. There is ample precedence for this approach to analyze genomic regions of genes implicated in human disease. Mutations in those conserved elements were found to cause human genetic syndromes, for example *SALL1*/Townes-Brocks syndrome³⁹ or *SHH*/preaxial polydactyly⁴⁰. Other groups have investigated the non-coding regulatory elements within disease genes such as *RET* (Ret proto-oncogene) and *MECP2* (Methyl-CpG binding protein 2) and found multiple regulatory enhancer and silencer elements^{38,41}.

Transcriptional regulation of dopaminergic neurons

Specific TFs seem to be directly involved in neurodegeneration and models of PD. TFs have been shown to be critical regulators for the development, maintenance and survival of dopaminergic

neuronal populations^{42,43}. E.g. forkhead transcription factor (*Foxa2*) is responsible for early development of endoderm and midline structures. *Foxa2* is specifically expressed in postmitotic dopaminergic neurons. Genetically engineered mice that are null for *Foxa2* are not viable, whereas heterozygotes for *Foxa2* develop major motor abnormalities starting at 18 months with an asymmetric posture, rigidity, and bradykinesia⁴⁴.

Conclusion

This screen of evolutionary conserved genomic elements in the *SNCA* locus showed a number of functionally elements that in an *in vitro* assay modulated the expression of a reporter gene. Furthermore, we identified very intriguing new candidate transcription factors that could directly regulate *SNCA* expression and could, if binding is altered by genetic variants, play a role in the pathological processes that underlie PD. This is the first step to systematically analyze the *SNCA* locus to understand its transcriptional regulation in more detail. Further studies are needed in neuronal tissues (e.g. dopaminergic neurons derived from patient-specific induced pluripotent stem cells) to confirm these findings and expand the analysis to identify *SNCA*-regulating transcription factors. By defining the transcription factors that regulate expression and potentially overexpression of α -synuclein that can lead to neurodegeneration, we will be able to identify targets for novel therapeutic approaches for α -synucleinopathies including Parkinson's disease.

Data availability

F1000Research: Dataset 1. Combined normalized raw datasets of Luciferase assays on *SNCA* conserved elements, [10.5256/f1000research.3281.d37452](http://dx.doi.org/10.5256/f1000research.3281.d37452)⁴⁵

Author contributions

BS conceived the study and designed the experiments, and drafted the manuscript. LS carried out the experiments. DT analyzed data. MW carried out *in silico* analysis for transcription factors. All authors were involved in the revision of the draft manuscript and have agreed to the final content.

Competing interests

No competing interests were disclosed.

Grant information

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Schüle, B., Sterling, L., Langston J.W.: Characterization of cis-regulatory elements in the alpha-synuclein gene; (Abstract, <http://www.ashg.org/genetics/ashg07s/f21298.htm>). Presented at the Annual Meeting of the American Society of Human Genetics, October 23–27 in San Diego, CA, USA.

Supplementary Table

Supplementary Table. Primer sequences and design for cloned ncECRs.

ECRs in SNCA locus on chromosome 4				HindIII: CCCAAGCTT			
				XhoI: CCGCTCGAG			
				KpnI: CGGGGTACC			
				BglII: GGAAGATCT			
ECR	Length	Identity	Location	Primers	PCR product length	Ann. Temp	Restriction sites within PCR product
D1	146bp	78.10%	chr4:90833665-90833810	CGGGGTACC CACGAAATCGTGCCAAAAAT GGAAGATCTaagtcacaaggtcgaggcttt	601bp	60C	no RE
D2	239bp	74.50%	chr4:90833665-90833810	CGGGGTACC tgcgaaattccacacaacat GGAAGATCTTACGAGATGGCATGGAATA	584bp	60C	no RE
D3-1/2	143bp	72%	chr4:90848813-90848955	CGGGGTACC AAGGGCTGACATTGGAATTG GGAAGATCTCCGCCTCTGAAAATAAGCAA	989bp	60C	no RE
D4	110bp	73.60%	chr4:90850858-90850967	CGGGGTACC GATGCAGCCATCAACTCTGA GGAAGATCTtggtgtagGCAGGAGAAATG	944bp	60C	no RE
D5-1	241bp	75.90%	chr4:90853634-90853874	CGGGGTACC ACTTCCTTGGGTAGGCGAAT			BglII at 1143
D5-2	114bp	75.40%	chr4:90854429-90854542	CCGCTCGAG GCTGAGATCACGCCACTGTA	1258bp	60C	use XhoI site
D6-1/2	234bp	83.30%	chr4:90855871-90856104	GGAAGATCTCCATTCCCTCACCTCAAATG CGGGGTACC TCTGCATGAATGTGCAAACA	582bp	60C	
D7	167bp	72.50%	chr4:90859690-90859856	GGAAGATCTggggctgtagtggaaatc CGGGGTACC GGGCAGTGCATACTTGTCTCT	856bp	60C	no RE
D8-1/2	100bp	75%	chr4:90860722-90860821	GGAAGATCTAGCTTCTGCCTTGTGTCTCC CGGGGTACC TTGAAGAACCCAAAATGCAA	1061bp	59C	no RE
I1	192 bps	81.80%	chr4:90871989-90872180	CCGCTCGAG aggataggctccaaccacct CGGGGTACC CAAATTCGGATCACGTAGGG	840bp	60C	BglII at 571 use XhoI site
I2	154bp	74%	chr4:90878220-90878373	GGAAGATCTcaggaattGGTGCAAAATCA CGGGGTACC aggggctgacctcaagatt	393bp	60C	
I3-1/2	276 bps	77.50%	chr4:90887100-90887375	GGAAGATCTtgaatgtgatggttcagcaaa CGGGGTACC gggaaggcaccctctaggtata	986bp	60C	no RE
I4-1/2	194 bps	75.80%	chr4:90891860-90892053	GGAAGATCTCCACCCCTCCACTTGACATA	899bp	60C	no RE

ECRs in SNCA locus on chromosome 4				HindIII: CCCAAGCTT			
				XhoI: CCGCTCGAG			
				KpnI: CGGGGTACC			
				BglII: GGAAGATCT			
ECR	Length	Identity	Location	Primers	PCR product length	Ann. Temp	Restriction sites within PCR product
	100 bps	75.00%	chr4:90892381-90892480	CGGGGTACC GCAATGGAAGTGTGGTGATG			
I5-1/2	109 bps	76.10%	chr4:90893684-90893792	GGAAGATCTCAGGCATGATTCTCCCTTA	705bp	60C	no RE
	155 bps	73.50%	chr4:90893990-90894144	CGGGGTACC CCATCAACATCCCAAGAACA			
I6	130 bps	74.60%	chr4:90894785-90894914	GGAAGATCTccttggtgattcctgaacat	355bp	60C	no RE
				CGGGGTACC GAAGTTGCCTGAGCTCCAAT			
I7	187 bps	75.90%	chr4:90897558-90897744	GGAAGATCTAGATGATGAGCAGGCAGTCC	432bp	60C	no RE
				CGGGGTACC cgaaccatagtgaaatcagg			
I8	112 bps	76.80%	chr4:90901290-90901401	CCGCTCGAG aaggcttgattggacattgc	474bp	60C	BglII at 34
				CGGGGTACC ctggaagaattggccacaa			use XhoI site
I9	199 bps,	75.40%	chr4:90906237-90906435	GGAAGATCTTGCAATGAAAACCACAATGG	561bp	60C	no RE
				CGGGGTACC tgttatgttctgtattccaccaa			
I10	269 bps	74.30%	chr4:90926832-90927100	GGAAGATCTtgggatgggtgggtaaatAG	899bp	60C	no RE
				CGGGGTACC tgtgtcaaggatGGAAAAAG			
I11	108 bps	74.10%	chr4:90929480-90929587	GGAAGATCTtcaaagcaagattttctcca	429bp	60C	no RE
				CGGGGTACC tggctccttttagcccaattt			
I12	255 bps	77.30%	chr4:90940532-90940786	GGAAGATCTagggagaggaaaaagcttgg	669bp	60C	no RE
				CGGGGTACC AAGGTTGAAAAACCGTGGTG			
I13	127 bps,	75.60%	chr4:90945579-90945705	CCGCTCGAG aggctctgggaccacaatta	578bp	60C	BglII at 328
				CGGGGTACC CCTCTTAACTTCTGGGCAACC			use XhoI site
I14	100 bps	75.00%	chr4:90958054-90958153	GGAAGATCTtccacctagaaccttacagga	701bp	60C	no RE
				CGGGGTACC ACACTTGAGTGTATGGACCCTCT			
I15	329 bps	76.30%	chr4:90961895-90962223	GGAAGATCTtcaacgttggtgacacctca	490bp	60C	no RE
				CGGGGTACC ccaGATAAATGCCATGCAAA			
I16	106 bps	75.50%	chr4:90976615-90976720	GGAAGATCTCCCGTTACCACCTGTTGACT	651bp	60C	no RE
				CGGGGTACC gccattcgacgacaggttag			
U1	261 bps,	81.60%	chr4:90977921-90978181	GGAAGATCTCCGTCCTCTCTCTCTAGTC	883bp	60C	no RE
				CGGGGTACC ATCACGCTGGATTTGTCTCC			
U2-1	105 bps,	76.20%	chr4:90980743-90980847	GGAAGATCTTTCATGTTTTGTTTTCTTTGCT	860bp	59.5C	no RE
U2-2	100 bps,	75.00%	chr4:90981402-90981501	CGGGGTACC caccagagttgcagagttgc			

ECRs in SNCA locus on chromosome 4				HindIII: CCCAAGCTT			
				XhoI: CCGCTCGAG			
				KpnI: CGGGGTACC			
				BglII: GGAAGATCT			
ECR	Length	Identity	Location	Primers	PCR product length	Ann. Temp	Restriction sites within PCR product
U3	329 bps,	73.90%	chr4:91004670-91004998	CCGCTCGAG ccatgcagttttccCCAATA	751bp	60C	BglII at 487
				CGGGGTACC TCTCTCTCATTTTTGGTTTTGACA			use XhoI site
U4-1			chr4:91,008,097-91,008,809	GGAAGATCTCTGAAGTAGGGGGCTCTTCC	535bp	60C	no RE
				CGGGGTACC GAGTTCTTTGGCAGGAGTGC			
U4-2	131 bps,	74.00%	chr4:91009155-91009285	GGAAGATCTggagaattcagttgctattgg	837bp	60C	no RE
				CGGGGTACC TGTGTTGCCATAGTCACATGTTT			
U4-3			chr4:91,010,061-91,010,758	GGAAGATCTAAGAAGAAGCAAGCCACACC	698bp	58C	no RE
				CGGGGTACC tttctgtagggtttatagtgcca			

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Jinglan Liu

Clinical and Molecular Cytogenetics, St. Christopher's Hospital for Children, Philadelphia, PA, USA

The article by Sterling *et al.* has described the identification and functional analysis of evolutionally conserved non-coding elements that might be involved in the transcriptional regulation of the gene *SNCA*, mutations in which were associated with Parkinson's disease. This is a very interesting, proof-of-concept article, with an attempt to provide pathogenic insight from the point of view of regulatory genomics for a complex human disease. I endorse the indexing of this manuscript.

It is now well recognized that ~98% of human genome do not code for proteins. Comparative genomics studies revealed that the majority of evolutionally conserved regions consist of non-coding elements that that might be involved in regulating gene expression. Genome-wide association studies (GWAS) have showed that the majority (~93%) of SNPs contributing to human diseases or susceptibility lie outside protein-coding regions, and there are many non-coding SNPs have been demonstrated to be associated with common diseases and traits.

By identifying functionally significant non-coding elements for *SNCA*, Sterling *et al.*'s work could lend a new perspective to study the genetic architecture of Parkinson's disease, and promote further investigations on the pathogenic impact of non-coding elements and their regulatory networks on the clinical courses of Parkinson's disease.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

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Ornit Chiba-Falek¹, Lidia Tagliafierro²

¹ Department of Neurology, Duke University, Durham, NC, USA

² Duke University, Durham, NC, USA

The paper by Dr. Schüle's team describes on the identification of evolutionary conserved non-coding regions (ncECRs) in the α -synuclein (*SNCA*) gene and their assessment as candidate regulatory

elements. The work coupled *in silico* and cell-based studies. By using a comparative genomic screen between human and mouse the authors identify 32 ncECRs, out of which 11 regions exert an effect on expression level using a luciferase reporter assay approach. Their findings add on previous reports in the field that have shown, using both luciferase reporter system and human brain tissues, that the SNCA gene contains cis-regulatory sites across the 3' and the 5' LD blocks that regulate its expression levels.

The study was well designed and thoroughly executed, the results are of interest to the scientific community of PD-genetics, and provide seeds for follow up studies. The paper is nicely written, logically flows and summarizes the literature in the field. However, the authors should make major revisions according to the following comments:

1. There is some inconsistency regarding the number of the ncECRs identified in the initial screen between the different sections of the article (32, 34, 37). Please make the corrections where needed.
2. Additional necessary control for the Luciferase experiments is a pGL-(SV40) promoter vector harboring an insert of a scrambled sequence that its size range mimics the average insert size of the tested ECRs. This is required to control for the 'spacer' effect of ECR lengths.
3. What method was used for the statistical analysis? It is also not clear in the text whether all significant changes were calculated in comparison to the SV-40 promoter-only vector. That should be described in details in the method section.
4. To demonstrate the important implication of this study the authors are recommended to follow up on an event as an example. That is to say, to evaluate the effect of a genetic variation, a PD-associated SNP, on the regulatory function of the corresponding ECR using the luciferase system established in this work. Figure 3 demonstrates overlap between PD associated SNPs and ncECR, connecting these dots will be of high significance.
5. Supp Table: there is a typo in the coordinates of D2. In the footnote include the human genome assembly of the coordinates.
6. Figure 2A X-axis: modify title to 'upstream...'
7. Omit Figure 3A. Instead include a new panel to figure 3B that indicates the position of the putative binding sites of these TFs within SNCA locus.
8. The identification of Transcription Factor Binding Sites (TFBS) is an important step required in order to evaluate the transcriptional regulation network of the SNCA gene. To this end, the computational prediction of TFBS is a classic approach that gives preliminary data but should be interpreted with caution. Integration of the classic approach with new models described in [Mathelier & Wasserman \(2013\)](#) is highly recommended. The relation between TF motifs and *in vivo* binding sites is far from simple. The analysis lacks of information about the context of the identified sequences. TF are highly context-specific, and the same TF typically binds to different genomic binding sites in different conditions. Obtaining information about the context could be helpful in better understanding the possible involvement of the predicted sites as TFBS. While this is beyond the scope of this study, this topic should be thoroughly discussed in the discussion section.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 26 Nov 2014

Birgitt Schuele,

We very much appreciate the careful review and excellent comments, suggestions and future directions of the reviewers. We hope to have addressed all of the comments to the reviewers' satisfaction.

There is some inconsistency regarding the number of the ncECRs identified in the initial screen between the different sections of the article (32, 34, 37). Please make the corrections where needed.

Thanks so much for the comment. We made changes to reflect the correct number of 34 ncECRs. We combined counts for ncECRs that were located very closely in the luciferase assay to one ncECR therefore different numbers appeared in the text. That has been addressed.

Additional necessary control for the Luciferase experiments is a pGL-(SV40) promoter vector harboring an insert of a scrambled sequence that its size range mimics the average insert size of the tested ECRs. This is required to control for the 'spacer' effect of ECR lengths.

We have included in our analysis three controls: 1. The pGL3-Basic Vector which lacks eukaryotic promoter and enhancer sequences should not show any transcription activity. 2. The pGL3-Enhancer Vector contains an SV40 enhancer located downstream of luc+ and the poly(A) signal and is showing transcription a very high levels (enhancer element is 246bp in length). 3) pGL3-Promoter Vector contains an SV40 promoter upstream of the luciferase gene (promoter is 202bp in length).

Even though we have not directly included a control with scrambled sequence, we think that the ncECR elements that do not change transcription of luc+ provide enough evidence that the experimental system is valid. Of a total of 34 in silico determined elements, only 12 show an effect of transcriptional regulation. 22 elements did not change expression compared to pGL3-Promoter Vector.

What method was used for the **statistical analysis**? It is also not clear in the text whether all significant changes were calculated in comparison to the SV-40 promoter-only vector. That should be described in details in the method section.

A description of the analysis of luciferase assays was lacking and has now been added as a paragraph at the end of Method section Cloning and luciferase assays and reads as follows:

“Statistical analysis:

Differences among means were analyzed using two-samples student's t-test. For differences in transcriptional activation of the luc+ gene, ncECRs were tested in quadruplicates in three independent experiments. Differences were considered statistically significant at $p < 0.05$.”

To demonstrate the important implication of this study the authors are recommended to follow up on an event as an example. That is to say, to evaluate the effect of a genetic variation, a PD-associated SNP, on the regulatory function of the corresponding ECR using the luciferase

system established in this work. Figure 3 demonstrates overlap between PD associated SNPs and ncECR, connecting these dots will be of high significance.

This is an excellent suggestion and will definitely be conquered in future work with this system as this is the basis for the understanding of transcriptional regulation of the SNCA locus for potential translational applications. The presented study was intended to understand the basic changes in transcriptional regulation within the SNCA locus.

Supp Table: there is a typo in the coordinates of D2. In the footnote include the human genome assembly of the coordinates.

We corrected the coordinates for D2 which was a duplicate of D1 with the correct genomic location chr4:90844830+90845413 and added in the header the corresponding Human Genome assembly NCBI36/hg18 (March 2006).

Figure 2A X-axis: modify title to 'upstream....'

Correction has been made. It reads now in Figure 2A "Upstream SNCA conserved elements". We also changed for consistency Figure 2B to "Intronic SNCA conserved elements" and capitalized Figure 2C "Downstream SNCA conserved elements".

Omit Figure 3A. Instead include a new panel to figure 3B that indicates the position of the putative binding sites of these TFs within SNCA locus.

We have modified Figure 3 according to the MatInspector network view with respective changes in the legend. We also included which genomic sequences have been analyzed in the text. Since this is a preliminary in silico analysis, we feel that the overview is sufficient and has to be validated in functional studies. As pointed out below by the reviewer, these analyses have to be taken with care and a grain of salt.

The identification of Transcription Factor Binding Sites (TFBS) is an important step required in order to evaluate the transcriptional regulation network of the SNCA gene. To this end, the computational prediction of TFBS is a classic approach that gives preliminary data but should be interpreted with caution. Integration of the classic approach with new models described in [Mathelier & Wasserman \(2013\)](#) is highly recommended. The relation between TF motifs and *in vivo* binding sites is far from simple. The analysis lacks of information about the context of the identified sequences. TF are highly context-specific, and the same TF typically binds to different genomic binding sites in different conditions. Obtaining information about the context could be helpful in better understanding the possible involvement of the predicted sites as TFBS. While this is beyond the scope of this study, this topic should be thoroughly discussed in the discussion section.

Thank you very much for this suggestion. Indeed, further studies are necessary to provide experimental evidence for the binding of predicted transcription factors. The analysis provided in this article was only a first step to model potential transcription factor binding sites and should stimulate further studies.

The reference Mathelier and Wasserman has been now included in the Discussion of the manuscript and reads as follows:

"Computationally determining transcription factor binding sites is a challenging process and multiple prediction algorithms have been developed over the last decade (Cartharius 2005, Wu 2009, Mathelier 2013). Therefore our preliminary data should solely open the discussion and drive novel hypotheses for potential transcription factors that regulate transcription of the SNCA locus."

Competing Interests: None.

