Regulation of alpha-synuclein expression in alcohol-preferring and -non preferring rats

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Abstract

The α-synuclein (*Snca*) gene is expressed at higher levels in alcohol-naïve, inbred alcohol-preferring (iP) rats than in alcohol-non preferring (iNP) rats. *Snca* modulates dopamine transmission and the dopamineregic system, which play a role in mediating the rewarding properties of alcohol consumption. Thus, understanding regulation of *Snca* gene expression could provide insight into the relationship of *Snca* and alcohol consumption. To study regulation of rat *Snca* expression, 1912 bp of the iP and iNP 5′-regions were cloned and sequenced. 5′-rapid amplification of cDNA ends (RACE), primer extension and RT-PCR mapped three transcription start site clusters (clusters TSS1, TSS2 and TSS3), suggesting that the *Snca* proximal promoter region has a complex architecture. This proximal promoter region has three TATA-less core promoters containing SP1 binding sites, initiator elements and downstream core promoter elements, which are often located in such promoters. *Snca-luc* constructs transiently transfected into SK-N-SH neuroblastoma cells showed that the region from − 1912 to − 1746 contained a strong core promoter, and that the entire approximately 2 kb region had significant promoter activity. Five polymorphisms identified between the iP and iNP in the proximal promoter region did not influence differential expression between the strains. In contrast, a single nucleotide polymorphism (SNP) at + 679 in the 3′-untranslated region (UTR) resulted in a 1.3-fold longer half-life of iP mRNA compared with iNP mRNA, which is consistent with the differential expression observed between the iP and iNP strains. These results suggest that regulation of rat *Snca* gene expression is complex and may contribute to alcohol preference in the iP rats.

Keywords

alpha-synuclein; expression; gene regulation; mRNA stability; polymorphism; rat selected strains

Alpha-synuclein (SNCA) belongs to a family of structurally related proteins that are predominately expressed in the central nervous system and are particularly abundant in presynaptic nerve terminals (Maroteaux *et al.* 1988; Iwai *et al.* 1995; Mori *et al.* 2002). Studies have demonstrated that SNCA might be important for the regulation of dopamine function. It has been implicated in neurodegeneration of dopamine neurons, especially in Parkinson's disease (Xu *et al.* 2002). However, it also appears to have a role in the normal

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brain. Studies have demonstrated the participation of SNCA in dopamine synthesis, storage, release and reuptake (Conway *et al.* 2001; Lee *et al.* 2001; Perez *et al.* 2002; Wersinger and Sidhu 2003; Sidhu *et al.* 2004; Yavich *et al.* 2004), as well as possibly serving to integrate presynaptic signaling and membrane trafficking (Zhu *et al.* 2003).

SNCA has been implicated in the etiology of several neurodegenerative disorders, including dementia with Lewy bodies, multiple system atrophy and Parkinson's disease (Mezey *et al.* 1998; Spillantini *et al.* 1998; Burn and Jaros 2001). Recently, *SNCA* mRNA and plasma protein levels were shown to be elevated in alcoholic patients and have been linked to craving (Bonsch *et al.* 2004, 2005a). The mesolimbic dopamine system projecting from the ventral tegmental area (VTA) to the nucleus accumbens has been hypothesized to mediate some of the reinforcing actions of ethanol, and dopamine levels have been shown to be lower in key limbic structures of P rats compared with NP rats (McBride *et al.* 1995). To date, however, the role of *Snca* regarding dopaminergic function and dysfunction remains unclear.

Employing multiple molecular approaches, including quantitative trait locus (QTL) analysis, total gene expression analysis (TOGA; Sutcliffe *et al.* 2000) and rats selected for alcohol preference (Li *et al.* 1991), *Snca* was recently identified as an interesting candidate gene that might influence the drinking behavior of the inbred alcohol-preferring (iP) and -non-preferring (iNP) rat strains (Liang *et al.* 2003). A genome screen of iP X iNP F2 animals identified a QTL on chromosome 4, which produced a highly significant logarithm of the odds score of 9.2 that accounted for 10% of the phenotypic, and approximately 30% of the genetic variation in alcohol consumption (Bice *et al.* 1998; Carr *et al.* 1998). *Snca* mapped to this chromosome 4 QTL. Sequence analysis identified a nucleotide exchange in the iNP 3′-untranslated region (3′-UTR) that reduced expression of the luciferase reporter gene in cultured SK-N-SH neuroblastoma cells. Expression studies indicated that Snca was expressed in the hippocampus at more than twofold higher levels in the iP than in the iNP rats (Liang *et al.* 2003). Immunohistochemistry studies demonstrated that protein levels of Snca in the hippocampus, septum and medial prefrontal cortex were significantly higher in iP rats compared with iNP rats (unpublished data). Interestingly, SNCA levels are elevated in midbrain dopamine neurons in chronic cocaine abusers (Mash *et al.* 2003) and in mice withdrawn from chronic morphine treatment (Ziolkowska *et al.* 2005).

The mouse (AF163865) and human (AF163864) *Snca* genes have been sequenced, and comparison of the sequences revealed that the exon/intron structures are highly conserved between the two species (Touchman *et al.* 2001). To date, few studies have been published concerning the genomic structure of the rat *Snca* gene and the regulation of its expression. The purpose of this study was to evaluate further the regulation of *Snca* expression in the iP and iNP rats. To achieve this, the nucleotide exchange, previously identified in the iP/iNP 3′-UTR, was tested for its affect on mRNA stability. Next, the 5′-region between – 1912 and + 1, relative to the translational start site, was cloned and sequenced, and polymorphisms were identified between the iP and iNP strains. Three clusters of transcriptional start sites (TSSs) were mapped, and deletion analysis of the 5′-region was carried out to assess promoter activity in *Snca* expressing neuronal and non-neuronal cell lines, and to determine whether the identified promoter polymorphisms were biologically significant.

Materials and methods

mRNA stability assay

The construction of the P2 and NP2 reporter plasmids used in this 3′-UTR stability study were described in Liang *et al.* (2003). Briefly, the + 642 to + 944 3′-UTR fragments, relative to the translation start site from iP and iNP containing the single nucleotide polymorphism (SNP) at + 679, were ligated between the luciferase gene and the SV40 late poly(A) signal of the pGL3-promoter vector (Promega, Madison, WI, USA). The P2 and NP2 luciferase plasmids were mixed with the Tfx-50 reagent and then transiently transfected into SK-N-SH cells in triplicate as described below (under Transient transfection and dual-luciferase assays), except that 1.5×10^6 cells were plated into each well of a 12-well plate. Twenty-four hours after transfection, the medium was replaced with fresh medium containing 100 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) to inhibit transcription. Following the addition of DRB, cells were harvested at 0, 30, 60 and 120 min, and total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. Genomic DNA contamination was removed using the DNA-free kit (Ambion, Austin, TX, USA), and the relative RNA levels were determined by quantitative RT-PCR using the ABI PRISM 7700 sequence detection system (ABI, Wellesley, MA, USA). Each reaction contained cDNA template that was reverse-transcribed from 35 ng of total RNA, 100 nM of forward and reverse primers, and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The luciferase forward LC527 (5′-CCTGGAACAATTGCTTTTACAGATG-3′) and reverse LC528 (5′-AAGAGAGTTTTCACTGCATACGACG-3′) primers were selected using VECTOR NTI ADVANCE Software (Invitrogen, Carlsbad, CA, USA). The PCR conditions were 95°C for 5 min, followed by 95°C for 15 s and 60°C for 1 min for 40 cycles. The cycle threshold (Ct) was determined for each cDNA template. The cycle threshold refers to the cycle number at which the fluorescence of the amplified product reached an arbitrary threshold that was within the exponential phase of amplification. The relative quantity of remaining luciferase mRNA at 0, 30, 60 and 120 min after DRB administration was calculated using the delta-delta Ct method (Livak and Schmittgen 2001). These values were normalized to the Ct values of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a standard 'housekeeping' control gene, using the human GAPDH forward primer, LC615 (5′-CAAGCTCATTTCCTGGTATGACAACG-3′), and the human GAPDH reverse primer LC616 (5′-AGGGTCTCTCTCTTCCTCTTGTGCTC-3′) for PCR amplification. All time points were performed eight times in triplicate using plasmids that were independently purified at least twice. The half-life of *Snca* mRNA was calculated using the decay formula $Nt = 2^{-t/x} \times N_0$ (Nt: different time point RNA amount, t: time, x: half-life, and N_0: the amount of RNA at time point 0). Comparison of the half-life from the P and NP transcripts was performed using the paired Student's *t*-test.

Northern blot assay

For the northern blot assay, a positively-charged nylon membrane from ZYAGEN (San Diego, CA, USA) had 20 μg total rat brain RNA per lane. *In vitro*-transcribed cRNA was used as the probe, and 405 bp of the *Snca* coding region and 224 bp of the 3′-UTR were cloned into the pCR4-TOPO vector. The plasmid was linearized, and 1 μg DNA template

was used to perform *in vitro* transcription with T3 RNA polymerase to obtain labeled antisense cRNA, according to the DIG RNA Labeling Kit protocol (Roche, Indianapolis, IN, USA). The northern blot assay was carried out using the DIG Northern Starter Kit (Roche), according to the manufacturer's instructions. Briefly, the blot was pre-hybridized for 30 min, followed by hybridization with 100 ng/mL probe in DIG Easy Hyb buffer at 68°C for 18 h. Two washes were performed in 2× sodium chloride-sodium citrate buffer (SSC), 0.1% sodium dodecyl sulfate (SDS) for 5 min at room temperature (25°C), followed by two high stringency washes in 0.1× SSC, 0.1% SDS buffer for 15 min at 68°C. After the washes, the signal was detected using anti-digoxigenin-AP and CDP-Star chemiluminescent substrate exposed to Lumi-chemiluminescent film (Roche Molecular Biochemicals, Indianapolis, IN, USA).

Identification of transcription start sites

The animals used in these experiments were maintained in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All research protocols were approved by the Institutional Animal Care and Use Committee, and are in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute on Drug Abuse, NIH, and the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council 1996).

Rats at 7 weeks-of-age were killed at approximately 09.00 hours and total brain RNA was isolated from one iP and one iNP rat using the RNeasy lipid tissue midi kit (Qiagen). TSSs were identified using FirstChoice RNA ligase mediated-rapid amplification of cDNA ends (RLM-RACE; Ambion) according to the manufacturer's protocol, which consisted of a reverse transcription reaction and two PCR reactions. In brief, full-length mRNAs were selected by treating total rat brain RNA with calf intestinal phosphatase (CIP), followed by treatment with tobacco acid pyrophosphatase (TAP) to remove the cap structure from the full-length mRNAs. A 45 bp RNA adapter was ligated to the uncapped, full-length mRNA, random decamers were annealed to the mRNA, and reverse transcription reactions were performed using M-MLV reverse transcriptase to generate the cDNA used in subsequent PCR. In the primary PCR, the outer adapter primer, identical to a portion of the 5′-adapter sequence, and an *Snca* gene-specific primer, LC583 (5′-TTGCTCTTTGGTCTTCTCAGCCACT-3′), complementary to sequences in the third exon, were used (Fig. 3a). PCR conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1.5 min, and a final extension of 72°C for 7 min. In the nested PCR using 2 μL of the primary PCR product as template, the inner adapter primer and a nested *Snca* gene-specific primer, LC582 (5′-TCTTTTGTCTTCCCAGCTGCCTCT-3′), complementary to sequences in exon two, were used. Primary and nested PCR products were analyzed on a 2% agarose/ethidium bromide gel. Major products from the nested PCR reaction were ligated into the pCR2.1-TOPO vector (Invitrogen) and sequenced with the Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, OH, USA). Using cDNA made for the first 5′-RACE assay for the template, a second 5′-RACE assay was performed using the gene-specific primer, LC460 (– 1175 to – 1151), and the outer adapter primer in the

primary PCR (Fig. 3a). The gene-specific primer LC519 (– 1482 to – 1462) and the inner adapter primer were subsequently used for the nested PCR.

Primer extension assays

Primers LC638, LC639 and LC640 (Fig. 4) were end-labeled at the 5′ end with [γ-^{32}P] ATP and T4 polynucleotide kinase. To anneal the primers, each primer (1.0×10^6 cpm) was mixed with 20 μg total RNA isolated from iP or iNP whole brain, or 5 μg total RNA isolated from transfected SK-N-SH cells and annealing buffer [40 m$_M$ 1,4-piperazinebis-(ethanesulphonic acid) (PIPES), 1 m$_M$ EDTA, 0.4 $_M$ NaCL, 80% deionized formamide]. The mixture was heated to 85°C for 10 min, 65°C for 10 min, then cooled slowly to 52°C before annealing at 52°C overnight. Primer extension reactions were performed at 50°C for 1 h in the presence of 1.0× reverse transcription buffer, 5 m$_M$ dNTPs, 5 m$_M$ MgCl$_2$, 10 m$_M$ dithiothreitol (DTT), 80 U RNase inhibitor and 400 U SuperScript III Reverse Transcriptase (Invitrogen). The extended products were resolved on a 6% polyacrylamide/8 $_M$ urea sequencing gel and compared with a sequencing ladder generated by the respective radio-labeled primer using the Thermo Sequenase Cycle Sequencing Kit (USB).

Plasmid construction

A set of 5′- and 3′- deleted reporter plasmids was constructed. To create the longest *Snca-luciferase* constructs, P6 and NP6, 1.9 kb DNA fragments were amplified with AmpliTaq DNA Polymerase (Applied Biosystems) from iP and iNP rat genomic DNA using the forward primer LC522 (– 1912 to – 1897; Fig. 4) and the reverse primer LC526 (– 34 to – 19; 5′-GCTCCACACTAGAGAA-3′) with *Mlu*I and *Bgl*II restriction sites incorporated into the 5′-ends of the forward and reverse primers, respectively. The plasmids were then digested with *Mlu*I and *Bgl*II, and the released fragments (– 1912 to – 19) were directionally ligated into the pGL3-basic vectors to create P6 and NP6 (Fig. 7). The plasmids P5 and NP5 were constructed by digesting P6 and NP6 with *Xba*I (– 703), treating with Klenow to blunt the ends, digesting with *Mlu*I (– 1912), and directionally cloning the gel-isolated 1.2 kb restriction fragment into the *Mlu*I and *Sma*I (blunt end) sites of the pGL3-basic vector. The plasmids P4 and NP4 were constructed by linearizing P6 and NP6 with *Avr*II, digesting with *Bgl*II to remove the – 1382 to – 19 fragments, and re-ligating the restricted plasmids that had been treated with Klenow to produce blunt ends. The plasmids P7 and NP7 were constructed by digesting P5 and NP5 with *Avr*II *(*– 1382) and *Mlu*I (– 1912), removing the – 1912 to – 1382 fragments, and re-ligating the restricted plasmids. The plasmids P3 and NP3 were constructed by amplifying fragments from iP and iNP rat genomic DNA using the forward primer LC522 and the reverse primer LC550 (–1746 to –1761; Fig. 4). These restriction fragments were directionally cloned into the *Mlu*I and *Bgl*II sites of the pGL3-basic vector. All the constructs were sequenced to ensure that there were no PCR errors incorporated in the *Snca* sequences.

Transient transfection and dual-luciferase activity assays

Human neuroblastoma SK-N-SH cells were cultured in Minimal Essential Medium (Invitrogen) containing 2 m$_M$ Glut-max, 0.1 m$_M$ non-essential amino acids, 1 m$_M$ pyruvate and 10% fetal bovine serum (FBS) (Invitrogen), and maintained at 37°C in a humidified 5%

CO_2 incubator. Twenty-four hours before transfection, 5.0×10^4 cells were plated into each well of a 24-well plate. Using Tf_x-50 reagent (Promega), 0.5 µg pGL-3 luciferase test plasmid along with 4 ng cytomegalovirus Renilla luciferase vector (pRL-CMV), to serve as an internal control for transfection efficiency, were co-transfected per well. Cells were incubated for 24 h, washed, and harvested using passive lysis buffer. Cell extracts were assayed for firefly and Renilla luciferase activities in a TD-20/20 Luminometer (Turner Biosystems, Sunnyvale, CA, USA), using the Dual-Luciferase Reporter Assay System (Promega). Assays were performed five times in triplicate using plasmids that were independently purified at least twice. Similarly, human cervical carcinoma HeLa cells were cultured and transfected three times in triplicate with the same set of *Snca-luc* constructs, using Tf_x-20 reagent (Promega). A two-way ANOVA was performed on the promoter constructs and genotypes; the least significant difference (LSD) test was used for pairwise posthoc comparisons.

Results

Polymorphism in the 3′-UTR alters mRNA stability

In previous transient transfection assays, a polymorphism at + 679 in the 3′-UTR of the *Snca* cDNA of iP (AY550005) and iNP (AY550006) rats altered expression of the luciferase gene (Liang *et al.* 2003). To determine whether the mechanism by which this SNP influenced expression of *Snca* was due to altered mRNA stability, the half-life of the luciferase mRNA transcripts in SK-N-SH cells was measured. The P2 and NP2 reporter plasmids used in this 3′-UTR stability study were the same as those plasmids used in the previous activity study (Liang *et al.* 2003). The half-life of the P2 transcript was 51 ± 4.89 (mean $\pm$ SEM) min, while the half-life of the NP2 transcript was 35 ± 4.73 min (Fig. 1). Thus, the P2 transcript had a significant 1.3-fold longer half-life than the NP2 transcript in SK-N-SH cells ($p < 0.005$). These results suggest that the SNP at + 679 alters mRNA stability and may, in part, be responsible for the differential expression of *Snca* in the iP and iNP rats.

Polymorphism in the 5′-region of the *Snca* gene

The region from − 1912 to + 1, relative to the translation start site, was sequenced in the iP and iNP rats to determine whether a polymorphism in the 5′-region of the *Snca* gene affects expression differences observed between the iP and iNP rats. Five polymorphisms were discovered between iP (DQ163910) and iNP (DQ163911), while three additional polymorphisms were identified in which the sequences of the iP and iNP were identical, but differed from the published rat genomic sequence (NM_047693) (Table 1). Because the genomic structure of the rat *Snca* gene has not yet been determined, the TSSs were mapped in the rat *Snca* gene to test whether these polymorphisms were functional and located in the promoter region or 5′-UTR.

Identification of exon 1 and determination of the TSSs of the rat *Snca* gene

Northern blot analysis of rat brain produced a prominent broad band from approximately 1.3–1.6 kb and a lighter band from 1.6–1.75 kb, suggesting that multiple *Snca* mRNA transcripts were being generated (Fig. 2). To determine if this was the case and to identify the TSSs of the rat *Snca* gene, 5′-rapid amplification of cDNA ends (5′-RACE) was

performed using total brain RNA from the iP and iNP rats. The primary PCR, using LC583 (Fig. 3a) and the outer adapter primer, produced a 400 bp band and a smaller band corresponding to the primers of the reaction (Fig. 3b). The subsequent nested PCR, using LC582 and the inner adapter primer, produced a major band of approximately 300 bp with a larger faint band at approximately 700 bp (Figs 3a and b). The 300 bp fragment was cloned and the initiation sites of 16 sequenced clones were determined (Fig. 4). The results, represented by different 5′-RACE clones, indicated numerous TSSs, which we will refer to as a cluster and which are commonly seen in TATA-less promoters. The TSS1 cluster extended from − 1322 to − 1292. Alignment of this cloned 300 bp fragment with the rat *Snca* genomic sequence revealed that it included part of the published exon 1 sequence (Exon 2 in Fig. 3a) and also, 162 bp that spanned from − 1305 to − 1144 of the genomic sequence (AF 007758 and NM_047693). This suggested that there was an intron in the genomic sequence from − 1144 to − 26 that was flanked by the canonical gt–ag splice-site sequences (Fig. 4; iP/DQ163910 and iNP/DQ163911). To verify that the sequences of the 300 bp fragment flanked an intron, PCR was performed using the forward primer LC461 and the reverse primer LC468 (Fig. 4), with iP and iNP cDNA and genomic DNA as templates. As expected, the PCR products generated from the cDNA were 113 bp whereas the products from the genomic DNAs were 1231 bp (Fig. 3c). These results further indicated that there was an intron in this 5′-region along with a non-coding exon in the rat *Snca* gene, which will now be designated as exon 1 (Fig. 3a). Furthermore, exon 1 in the published rat *Snca* sequence will now be designated as exon 2. Thus, the TSS1 cluster was mapped from − 1322 to − 1292 upstream of the translational start site of the rat genomic DNA sequence. Although none of the cloned fragments from the 5′-RACE assay was larger than 300 bp, it appeared from the gel that there was a larger, less abundant PCR product (Fig. 3b, lanes 2 and 4). Therefore, a second 5′-RACE was carried out using gene-specific primers located in the newly discovered exon 1 to determine if there was an additional TSS. The gene-specific primer, LC460 (Fig. 3a), and the outer adapter primer were used in the primary PCR, and the inner adapter primer and the gene-specific primer, LC519 (Fig. 3a), were used in the nested PCR. The template was cDNA generated from the initial 5′-RACE experiment. The primary PCR produced a band approximately 200 bp in length, and the nested PCR produced two distinct bands approximately 110 bp and 220 bp in length (Fig. 2d). From the 110 bp fragment, three clones indicated that the TSS was at − 1533. From the 220 bp fragment, initiation sites of 14 sequenced clones were determined and most of them mapped between − 1636 and − 1641; the most 5′ initiation site was at −1692 (Fig. 4). To confirm the TSS2 cluster, genomic DNA and cDNA were amplified in parallel using the forward primer, LC612 (Fig. 3a), and the reverse primer, LC468 (Fig. 3a). As expected, a 524 bp fragment was generated from the cDNA template (Fig. 3e) and a 1.64 kb fragment was generated from genomic DNA (data not shown), confirming that the region downstream of − 1641 was part of the *Snca* cDNA.

A search of the promoter prediction database http://www.fruitfly.org/seq_tools/ promoter.html revealed that there was a predicted promoter in the region from − 1900 to − 1850. There are many repetitive sequences 5′ of the TSS2 cluster that might form stem-loop structures and prevent the reverse transcriptase enzyme from reaching a more distal TSS. Thus, to determine whether a TSS might be upstream of the TSS2 cluster and near the

predicted promoter, we designed a set of forward primers that covered the region from − 1930 to − 1692. The primers LC516 and LC627 (Fig. 3a) were the two most 5′ primers tested. Using these primers and the reverse primer, LC468, to amplify cDNA and genomic DNA revealed that LC516/LC468 produced a 1947 bp PCR product with the genomic template, but no product with the cDNA template, while LC627/LC468 produced a 777 bp product with the cDNA template and a 1894 bp product with genomic DNA as template (Fig. 3f). These data suggested that the most 5′-TSS3 cluster initiation site was located between − 1910 and − 1878, which would be consistent with the prediction that there is a promoter located in this region.

To evaluate further the TSS clusters identified by 5′-RACE, primer extension was carried out using primers located in the new exon 1 (Fig. 4). Using total RNA from whole brain, primer LC640 produced numerous cDNA products that extended from − 1297 to − 1351 of the genomic sequence (Fig. 5a). These results were similar to, and confirmed, the TSS1 cluster from − 1292 to − 1311, identified using 5′-RACE. Using total RNA from SK-N-SH cells transfected with the P5-*luc* construct (Fig. 7), primer LC640 produced numerous cDNA products that extended from − 1291 to − 1332 (Fig. 5b), suggesting that this core promoter was active in the reporter assays. In addition, a cluster from − 1595 to − 1678, and a lighter cluster further upstream where the sequence could not be distinguished, were observed, suggesting that TSS2 and TSS3 were also active in the SK-N-SH cells (data not shown).

Because the data generated for primer extension are best if primers within 100–150 bp of the TSS are used, primer LC638, located upstream of the LC640 primer (Fig. 4), was employed. This primer also produced numerous cDNA products that extended from − 1594 to − 1659 of the genomic sequence (Fig. 4). This TSS2 cluster identified by primer extension overlapped and confirmed the TSS2 cluster (− 1631 to − 1692; 220 bp clones) previously identified by 5′-RACE. The TSS at − 1533 observed from sequencing the 110 bp clones was not confirmed. The TSS3 cluster identified by RT-PCR was confirmed using primer LC639 in the primer extension assay. Two prominent bands at − 1884 and − 1906 were observed (Fig. 5d), suggesting that the most 5′ initiation site was located at − 1906. For numbering purposes, nt−1305 was designated as TSS1, based on multiple RACE clones with identical 5′-ends that were close to those identified by primer extension (Fig. 4). The TSS2 designated site at − 1636 was based on an initiator element located within the TSS2 cluster, and the TSS3 site was positioned at − 1906 based on the primer extension data.

Taken together, 5′-RACE, primer extension and RT-PCR data suggested that there are three TSSs in the *Snca* gene.

Transcription binding sites in the 5′-region of the *Snca* gene

Binding elements that are common in TATA-less promoters were identified in the *Snca* 5′-region using the TRANSFAC database. The consensus sequence for a YY1 binding element, which has been shown to be a prominent initiator binding protein (Clark *et al.* 1998), was located within the TSS1 cluster. Consensus sequences for initiator elements (Inr; Py-Py-A_{+1}-N-T/A-Py-Py) and downstream core promoter elements (DPE; A/G-G-A/T-C/T-G/A/C) (Butler and Kadonaga 2002) were identified within the TSS2 and TSS3 clusters (Fig. 4). In

addition, six Sp1 sites, known to be associated with TATA-less promoters, were located in this region. Other interesting transcription factor binding sites are also indicated in Fig. 4. Human, mouse and rat 5′-region sequences were compared (AF 163864, AF 163865, and NW_047693, respectively) to identify conserved sequences that may play a role in regulating *Snca* expression (Fig. 6). High homology existed between the three species from rat − 1980 to − 1682, and from − 1576 to − 1292, suggesting that common regulatory mechanisms between the species might exist. Several binding sites were identical between the species, including TFIID, c-Est-2. NF-ATc, AP-1, GATA-1, kappa Y factor, Sp1 and Nf-s (Fig. 6). The rat TSS1 cluster overlapped the mouse TSS1. All three rat TSS cluster sequences were identical to the mouse sequences, but differed from the human sequences.

Transcriptional activity of the putative rat *Snca* gene promoters in transiently transfected SK-N-SH cells and HeLa cells

As a first study, several 5′- and 3′-deleted *Snca-luc* plasmids were constructed (Fig. 7a) to begin evaluation of the putative *Snca* promoters, and to test whether the identified 5′-region polymorphisms were biologically significant. All constructs were transiently transfected into human neuroblastoma SK-N-SH cells and human HeLa cells, which constitutively express *Snca*. Activities of the *Snca-luc* plasmids were expressed as fold changes compared with the promoterless luciferase reporter vector, pGL3-basic (Fig. 7b). A two-way ANOVA indicated that there was no interaction between the genotypes and the luciferase constructs ($p = 0.90$), and no significant effect between luciferase activity of each of the *Snca-luc* P plasmids, compared with activity of each of the corresponding *Snca-luc* NP plasmids (genotype effect) ($p = 0.25$; Fig. 7b). These results suggested that the polymorphisms in the region from − 1912 to − 19 were not playing a role in the differential expression of *Snca* previously observed between the iP and iNP animals (Liang *et al.* 2003).

The two-way ANOVA indicated that promoter activity between the deleted promoter constructs was highly significant ($p < 0.0001$). The promoter activities of the P3/NP3 and P6/NP6 constructs were significantly different from each other and from the other constructs; no significant differences were observed among the other constructs. Thus, the longest constructs, P6 and NP6 (− 1912 to − 19), exhibited significant promoter activity, with the luciferase activity being 3.02- and 3.6-fold higher than the pGL3-basic vector, respectively. Deletion of the 3′-region from − 19 to − 703 (P5 and NP5) significantly decreased activity. Further deletion to − 1382 (P4 and NP4) did not alter activity, but deletion to − 1746 (P3 and NP3) resulted in a large increase in promoter activity. The activity of P3 and NP3 was more than 11-fold higher than that of the pGL3-basic vector. Deletion of the 5′-region of P5 and NP5 to − 1382 (P7 and NP7) did not change promoter activity.

To search for promoter elements that might be involved in neural-specific expression, the same set of *Snca-luc* plasmids was transfected into HeLa cells. The activity observed with HeLa cells was very similar to that observed in SK-N-SH cells (data not shown), suggesting that tissue-specific elements were not located in this − 1912 to − 19 region and may be located farther upstream or elsewhere in the gene.

Discussion

In this study, 1912 bp of the 5′-regulatory region of the *Snca* gene from the iP and iNP rats were cloned and sequenced. Five polymorphisms were identified between the two rat strains in the − 1912 to − 19 region, but none appeared to alter expression when tested in luciferase reporter assays. Using northern blot, 5′-RACE, primer extension and RT-PCR assays, three TSS clusters were identified in this region. *Snca-luc* constructs transiently transfected into SK-N-SH neuroblastoma cells showed that the region from − 1912 to − 1746 contained a strong promoter (TSS3 core promoter) and that the entire approximately 2 kb promoter region had significant promoter activity. Very similar *Snca-luc* activity changes were observed when the same set of reporter constructs was transfected into HeLa cells, which suggested that the different TSS clusters and regulatory elements in this region are not responsible for tissue-specific expression but rather, that there are tissue-specific elements elsewhere in the gene.

The sensitive quantitative RT-PCR assay was used to determine luciferase mRNA levels in the mRNA stability assays, unlike studies that have used other methods such as RNase protection or northern blot to calculate mRNA half-life (Chrzanowska-Lightowlers and Lightowlers 2001). An SNP identified in the 3′-UTR resulted in mRNA with a longer half-life in the iP rats relative to the iNP rats (Fig. 1). This is consistent with previously collected data in which (i) the iP reporter construct exhibited twofold greater expression when compared with the iNP construct and (ii) Snca protein levels were 1.6 higher in iP in the caudate putamen and hippocampus compared with iNP (Liang *et al.* 2003). This is also consistent with other studies that have shown that eukaryotic mRNA-3′-UTR influences mRNA stability (Ross 1995; Lazarov *et al.* 1999; Nair and Menon 2000).

Evidence suggests that the regulation of mRNA stability involves the binding of *trans*-acting factors to *cis*-elements located in the 3′-UTR that can either be sequence-specific or promoted by stem-loops formed in the mRNA (Tillmann-Bogush *et al.* 1999; Cuadrado *et al.* 2002; Putland *et al.* 2002). These *cis*-elements serve to modulate the susceptibility of the mRNA to degradation. A search of the website http://www.genebee.msu.su/services/rna2_reduced.html revealed that the SNP at + 679 altered the secondary structure of iP mRNA compared with iNP mRNA, which could influence *trans*-acting factor binding. Thus, the 3′-UTR SNP appeared to play a role in the regulation of rat *Snca* expression, but to clarify whether this regulation is the result of sequence-specific binding, conformational change or both will require further studies.

The rat gene has been reported to have five exons, whereas the human and mouse genes both have six exons. Comparison of the human, mouse and rat genomic sequences (AF 163864, AF 163865 and NW_047693, respectively) revealed that the *Snca* gene is highly conserved between the three species, especially the mouse and rat. Sequence comparison between mouse and rat indicated that mouse exon 2 was 98% homologous with sequences designated as exon 1 in the published rat mRNA sequence (AF 007758). When the − 1912 to + 1 bp rat genomic sequence in this study was compared with the published rat mRNA sequence, it was evident that there was a non-coding exon in the rat similar to that reported in the mouse mRNA (AK 014472). Comparison of exon 1 sequences in mouse and rat with human also

revealed that these sequences are similar to human exon 1, but not to the human exon 1 splice variant designated human exon 1′ (Xia *et al.* 2001). From this study, and sequence alignment analysis with other species, the presence of a new exon 1 in the rat *Snca* gene was confirmed. Thus, rat *Snca* has six exons, making it similar in structure to the mouse and human genes.

For many genes, the core promoter element is the TATA box found about 25–30 bases upstream of the TSS. Analysis of the 1.9 kb sequences upstream of the TSSs, using the TATA signal prediction database (http://l25.itba.mi.cnr.it/~webgene/wwwHC_tata.html) allowing for no mismatches, revealed that the promoter regions of the rat *Snca* lacked a classical TATA box and a CCAAT box at the expected positions. TATA-less core promoters, as discovered in the *Snca* proximal promoter, often initiate from numerous start sites (cluster) that can be distributed over a region of about 50–100 nucleotides (Butler and Kadonaga 2002). In contrast, transcription from TATA core promoters occurs from a single site. TSS1 and TSS2 clusters were mapped using 5′-RACE and primer extension. The TSS3 cluster, identified by primer extension and RT-PCR, was not detected by 5′-RACE. It might not have been observed in the 5′-RACE assay because there are numerous repetitive sequences in this region that could potentially form secondary structures, which would limit the ability of the reverse transcriptase to reach the end of a long mRNA transcript, or the transcript was in low abundance so that it could not be isolated from a gel and cloned for further evaluation.

Identification of multiple TSS clusters in the rat *Snca* gene suggested that alternative promoter usage might exist. Alternative transcripts have been mapped in the mouse (NM_009221) and human (Ueda *et al.* 1994). Similar to rat, multiple 5′-RACE clones were also observed when determining the TSS in the human *SNCA* gene (Xia *et al.* 2001). The proximal mouse TSS (AK 014472) is located in a similar position to the rat TSS1 at − 1305, with a difference of approximately 6 bp (Okazaki *et al.* 2002; Fig. 6). Although the TSS clusters identified in this study were confirmed with primer extension, it is possible that RNA degradation or secondary structures could result in spurious results and thus, the TSS clusters might not be precise.

Studying transcriptional regulation of eukaryotic genes has resulted in unforeseen variety and complexity. Several sequence motifs, including the TATA box, Inr, DPE and TFIIB recognition element, are common in core promoter elements. However, each of these core promoter elements is found in some but not all core promoters; a TATA box was not observed in the three TSSs identified in the rat *Snca* promoter. In addition, increasing evidence exists for the presence of multiple promoters directing the transcription of a single gene, which appears to be the case in the rat *Snca* gene, and often, alternate promoters are associated with tissue-specific or developmentally-regulated gene expression (Schibler and Sierra 1987; Valdenaire *et al.* 1994).

Often, members of the Sp family of transcription factors activate transcription of TATA-less promoters. Use of the TRANSFAC database to search for transcription factor binding sites revealed that there were six Sp1 binding sites between TSS1 and TSS3 (Fig. 4). Inr elements are located within the TSS2 and TSS3 clusters. Inr elements encompass transcription start

sites and have been identified in a variety of eukaryotic genes (Breathnach and Chambon 1981). There is evidence that TFIID binds to the Inr element (Oelgeschlager *et al.* 1996) and, interestingly, there is a binding site for TFIID in the Inr located in TSS3 (Figs 4 and 6). It is also known that TFIID binds co-operatively to Inr and DPE motifs (Burke and Kadonaga 1996), and DPE motifs are located downstream of the TSS2 and TSS3 Inr elements. YY-1 binding sites within an initiator region have been shown to be sufficient for transcription initiation (Hariharan *et al.* 1989), and a YY-1 site is located in the TSS1 core promoter cluster. Other interesting putative transcription factor binding sites that were conserved between rat, mouse and human include AP-2, AP-3, AP-4, cMyb, cEBP, PU1, COUP, GATA-1 and Olf-1. Additional studies will need to be performed to determine whether these transcription factor binding sites are functional; even though the site is present, it may not be functional.

To begin testing how expression of the *Scna* gene was regulated by 5′ sequences, deleted *luc*-constructs were transfected into SK-N-SH and HeLa cells. The longest constructs (P6/NP6) had an approximately 3.5-fold higher activity than the pGL3-basic vector. Deletion of the 3′-region from − 19 to − 703 (P5/NP5) significantly decreased activity, suggesting that there are potential positive transcriptional binding sites in this intron region. A search of this region for transcription factor binding elements identified, among others, a GATA-1 site, AP-1, AP-2 and AP-4 sites, and three Sp1 sites. Others have proposed that long introns at the 5′-end of a gene contain regulatory elements that influence regulation of gene expression (Sobocki *et al.* 2006). Thus, this region may prove to be important in the regulation of rat *Snca* expression. Further deletion to − 1746 resulted in significant increases in activity. The P3/NP3 *luc*-constructs (− 1912 to − 1746) exhibited the highest activity, which was more than 11-fold higher than the activity of the pGL3-basic vector, suggesting that this region has strong promoter activity. These preliminary results suggest that this is a complex promoter that will need to be finely dissected to determine whether TSS1 and TSS2 core promoters are active in SK-N-SH and HeLa cells. It appears that the TSS3 core promoter has strong promoter activity and that the entire 5′-region with all three TSS clusters is active, but because of large blocks of genomic sequences that could bind enhancers or repressors surrounding the TSSs, it is not possible to determine whether each of these core promoters is functional in SK-N-SH and HeLa cells.

Although the polymorphisms analyzed between iP and iNP in the 5′- region were not functional in reporter assays, it is possible that, in addition to the functional 3′-UTR observed, there are additional functional polymorphisms further upstream, such as the NACP-Rep1 repeat (Chiba-Falek and Nussbaum 2001). Alleles at NACP-Rep1, the polymorphic microsatellite repeat located about 10 kb upstream of the human *SNCA* gene, modulate *SNCA* expression (Chiba-Falek *et al.* 2003) and have been associated with alcohol-dependent patients (Bonsch *et al.* 2005b) and increased risk of Parkinson's disease (Farrer *et al.* 2001; Tan *et al.* 2000). A search of the 5′-region of the rat *Snca* gene (NM_047693) revealed several repeat regions 20 kb upstream of exon 1. Whether these regions are polymorphic between iP and iNP rats, and are influencing *Snca* expression, which, in turn, is associated with alcohol seeking behavior, will be addressed in future studies.

Acknowledgements

Abbreviations used

DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
iNP	inbred alcohol-non-preferring
iP	inbred alcohol-preferring
RACE	rapid amplification of cDNA ends
SNCA	alpha-synuclein
SNP	single nucleotide polymorphism
TSS	transcriptional start site
UTR	untranslated region

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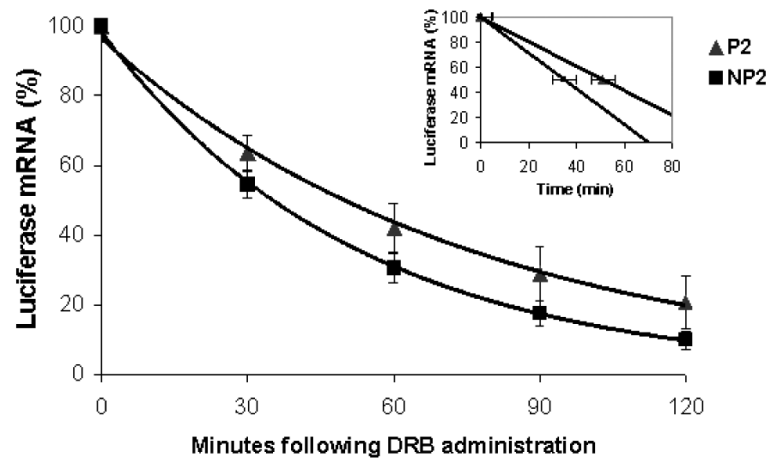
Fig. 1.
P2 luciferase mRNA is more stable than NP2 luciferase mRNA. Constructs P2 and NP2, created previously for transient transfection luciferase reporter assays (Liang *et al.* 2003), were used to transfect SK-N-SH cells. The relative amount of mRNA was quantified using quantitative RT-PCR. Luciferase mRNA decay rate was determined at 30, 60, 90 and 120 min following DRB administration; the mean and SEM are indicated. Inset: the average luciferase mRNA half-life times were 51 ± 4.89 min (mean ± SEM) and 35 ± 4.89 min for the P2 and NP2 constructs, respectively.
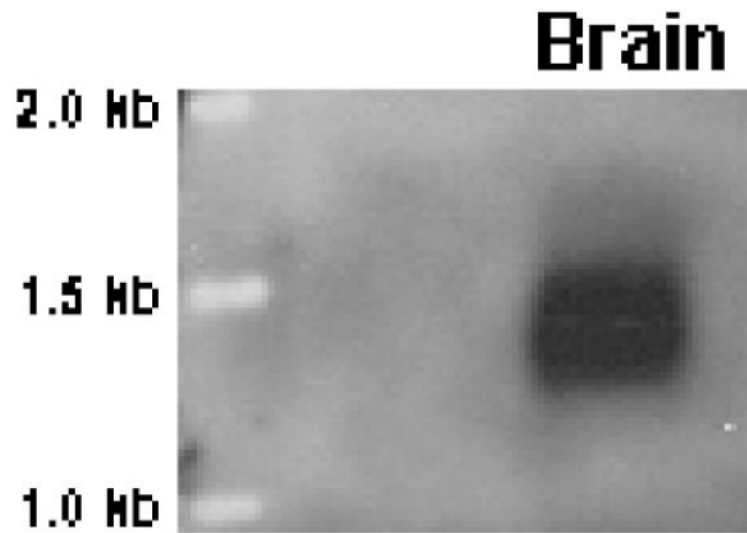
Fig. 2.
Northern blot of total RNA from rat brain. White lines on the blot are size markers and designate the sizes of the mRNA transcripts as indicated.
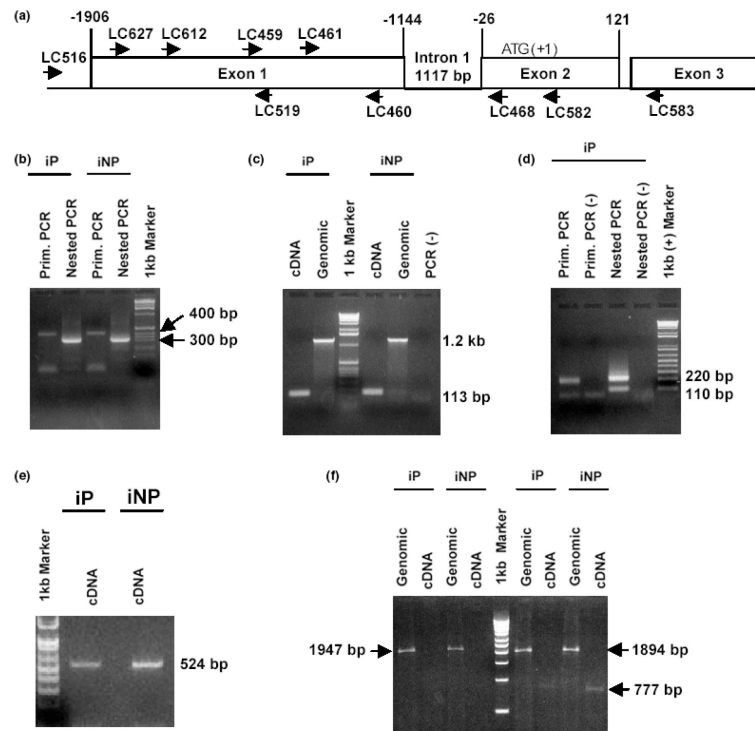
Fig. 3.

Identification of TSSs and Exon1. First Choice RLM-RACE was performed two times with different primer sets using total brain RNA from the iP and iNP rats. All the PCR products were run on 2% agarose gels. (a) Genomic structure of exons 1, 2 and 3, and intron 1, of the rat *Snca* gene. Open boxes represent exons, intron 1 boundaries are noted, and arrows represent forward and reverse primers used in the 5′-RACE assay and confirmation studies. The translation start site, ATG, is designated as + 1. (b) First 5′-RACE assay: 400 bp denotes the size of the primary PCR product in iP and iNP rats, using the 5′–-RACE outer adapter primer and LC583; 300 bp denotes the nested PCR product using the 5′-RACE inner adapter primer and the gene-specific nested primer LC582. A larger nested PCR product is barely visible at approximately 700 bp. The 300 bp fragment included part of the published exon 1 sequence (exon 2 in this paper) and also 162 bp of a new exon 1, which mapped from − 1305 to − 1144 of the genomic sequence. (c) Verification of exon 1 and intron 1. PCR was performed, using primers LC461 and LC468, with iP and iNP cDNA and genomic DNA as templates; (–) denotes a PCR reaction without template. (d) Second 5′-RACE assay. This assay was identical to the first 5′-RACE assay except that the outer reverse gene-specific primer was LC460 and the inner reverse gene-specific primer was LC519. The nested iP PCR products are noted at 110 bp and 220 bp; iNP data (not shown) were identical to the iP data. (e) Confirmation of TSS2. iP and iNP cDNA were amplified in parallel using primers LC612 and LC468 to confirm TSS2; 524 bp denote the size of the cDNA product. (f) TSS3. Forward primers LC516 and LC627, and reverse primer LC468, were used to amplify iP and iNP genomic DNA and cDNA. The LC627/LC468 cDNA product is 777 bp and includes 735 bp, indicated as exon 1 and 42 bp from exon 2. The genomic product is 1894 bp. The LC516/LC468 genomic PCR products are 1947 bp and there was no amplification, using cDNA for the template.

```
                            AP4              c-Myb        LC516       Inr        LC522      DPE
-1962 GTGCAAAAAA GCCTTTAGGC AGCTGGTTTT CGACGGTTGG GGTTTATTTA TTTCTTGCTC CACAGATGGG
                                                                            rTSS3
             Hb          LC627                        YY1
-1892 GGAAAAAAAA ATCAGCGTCT GGCAGCCGCT GATTGGTGGA AACGAAAATG GTGATAGTGG AGTGGGAAAG
                       LC525
                 Sp1                  COUP                                    Sp1
-1822 AGGATTTGCT GAGCCTCCCC CTGCCTCCTC GACCTGTAAC TCTTCCTTAG TCGGCTCCCC TTCACACCCA
                                                   LC639
          NF-Gma           LC518         AP-3                         c/EBP         1
-1752 GAAGAACCTT TTTAGACTCC TTCGGGGTTA AAACAAATGG AATTCTCAGG CTGTGTGAAC AAAAGCAACC
                   Sp1                          1          4 3  3   Inr  2      DPE    LC612
-1682 CGAAGGGTGT GTGCTCCCTC CCCTCGCCTG GCTCCGCACA CAGATCATTT CAGGCGGTTC AGTCCTCTGG
                                                                 rTSS2
              REB1         Sp1                   YY1                           PU1
-1612 TGTCCGCTTC CCGGCTCGTC CGGAGGAGGG GGGTCGCCTA GAGGAACCGA GAACAGGCTG AGGCAGGGAA
                LC613                                 RVF                 LC459
-1542 GAAGGGGATG AGATAGGAAA GAGGCCAGCT CAAGTTCAGC CACGATAAAA CCGAAGGCCC CCTGAACTCG
                                                                LC638
-1472 AGGTGAAACT CAGGCTACCC TCTCTTCCCT CCTCCTGGGG AGCGTGCTCC CGGTACCCAC AGCCCTCACG
        LC519                                      AP-2
                 Sp1           Olf-1                            Sp1
-1402 CACCGCCATC CCGTCCCCTC CCTAGGAAAA CGAGCGAAGG CACGAGGCAG GCGAGGGGCG GGGAGAGGAG
               2                    3 4                   3 1         3
-1332 CTGACAAATC AGCTGCGGGG GCGACGTGAA GGAGCCAGGG AGCCAGAGCG CCCGGCAGCA GACGGCAGGA
             AP-4          YY1          NF-ATp        GATA factor
         E12 / MyoD             Myogenin             rTSS1              LC461          c-Myb
-1262 GACCAGCAGG TGCTCCCCCT GCCCTTGCCC CTCAGCCCAG AGCCTTTCAC CCCTCTTGCA TTGAAATTAG
                   LC640
       C/EBP        PPUR
-1192 ATTGGGGAAA ACAGGAGGAA TCAGAGTTCT GCGGAAGCCT AGAGAGCCG gt............. ............agTGTG
                                                 LC460

  -22 GAGCAAAGAT ACATCTTTAG CCATGGATGT GTTCATGAAA
                          +1                     LC468
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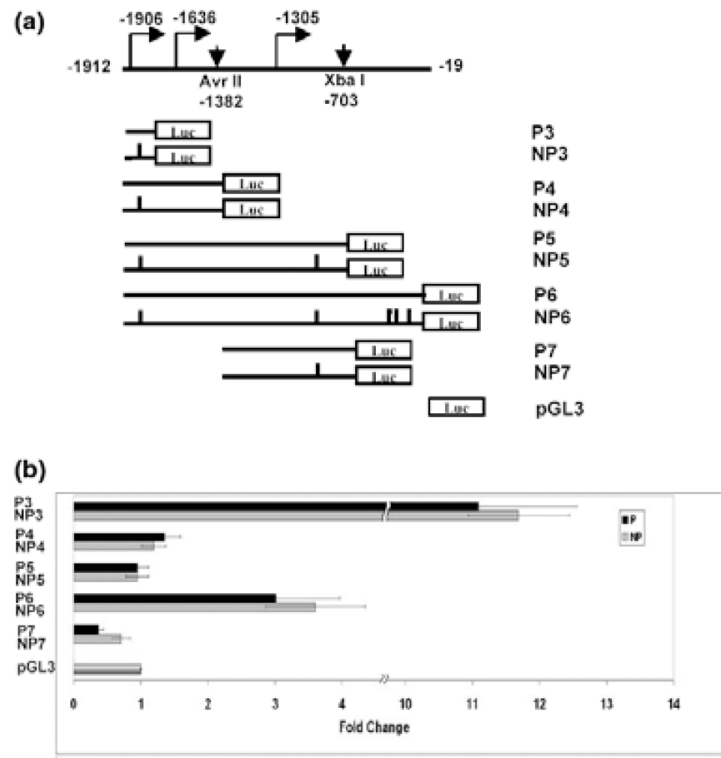
Fig. 4.

Nucleotide sequence of the rat *Snca* 5′-region. The translation start site is indicated as + 1. Initiation start sites (clusters) identified by 5′-RACE are designated by arrows and the number of clones above the sequence. The initiation start sites identified by primer extension are indicated by bold letters and underlining. For numbering purposes of subsequent figures, the three rat TSSs, rTSS1, rTSS2 and rTSS3, are indicated by a bold letter. Putative *cis*-acting elements are shaded in gray with the transcription factor above the sequence. Initiator (Inr) and downstream core promoter element (DPE) consensus sequences are boxed. Reverse primers and forward primers are shown as horizontal lines, with arrows below the sequence and above the sequence, respectively. Lower case letters represent intron canonical GT-AG splice-site sequences flanking intron 1.
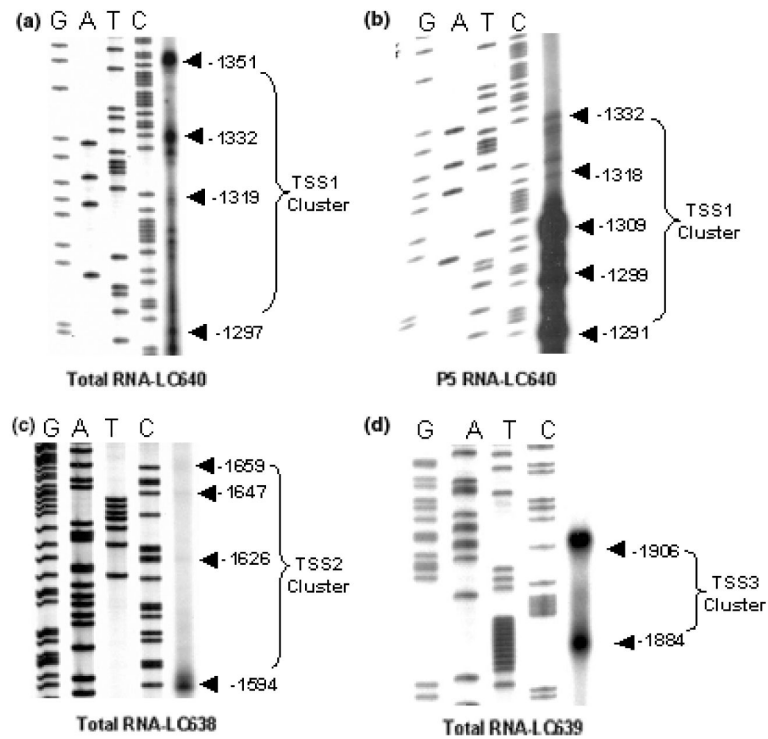
Fig. 5.
Primer extension assays confirm putative TSS clusters. Primers LC640, LC638 and LC539 (Fig. 4) were end-labeled with [γ^{32}P]-ATP and T4 polynucleotide kinase. Primers were annealed to total rat brain RNA in (a), (c) and (d), and to total RNA from SK-N-SH cells transfected with the P5-*luc* construct in (b). Extension products were resolved on a 6% sequencing gel and compared with a sequencing ladder generated by the respective end-labeled primers. Location of the primer extension products are indicated.

Fig. 6.

Comparison of 5′-region sequences in rat, human and mouse to identify conserved binding sites. The numbering is relative to the + 1 translation start sites. Binding sites identical between the species and binding sites, with mismatches that bind the transcription factor, are bold or not bold below the sequence, respectively. Sequences shaded in gray are identical between at least two of the species. Rat (rTSS), mouse (mTSS) and human (hMSS) transcription start sites are shown as arrows below the sequence.

Fig. 7.
α-Synuclein promoter activity in transient transfection assays. (a) A schematic diagram corresponds to the *Snca* gene from – 1912 to – 19 relative to translation start site (+ 1). Restriction sites are noted. Arrows indicate three putative transcription initiation sites within the three identified TSS clusters. Various deletion promoter fragments designated P3/NP3 to P7/NP7 from the iP and iNP 5′-regions were fused to the luciferase reporter gene. Vertical lines are polymorphic sites between the iP and iNP rats (see Table 1). (b) The activity of each construct was expressed as fold change compared with the activity of the pGL3-basic vector. The bars and fold change show the mean ± SEM of the results from at least five independent transfection experiments performed in triplicate.

Table 1

Scna sequence differences in the 5′-region

Name	Position (bp)							
	− 1890	− 1605	− 1490	− 1036	− 222 to − 219	− 201	− 183	− 144 to − 142
NW_047693	A	T	G	T	TCAA	A	T	TTT
iP rat (DQ163910)	A	G	–	T	TCAA	A	G	–
iNP rat (DQ163911)	AA	G	–	C	–	AA	G	TTTT