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Influence of Different Promoters on the Expression Pattern of Mutated Human α-Synuclein in Transgenic Mice

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Key Words
Parkinson's disease · Promoter activity · Nigrostriatal system · α-Synuclein · Transgene

Abstract
Two missense mutations (A53T and A30P) in the gene encoding the presynaptic protein α-synuclein (asyn) are associated with rare, dominantly inherited forms of Parkinson's disease (PD) and its accumulation in Lewy bodies and Lewy neurites. As an initial step in investigating the role of asyn in the pathogenesis of PD, we have generated C57BL/6 transgenic mice overexpressing the doubly mutated human asyn under the control of three different promoters; the chicken β-actin (chβactin), the mouse tyrosine hydroxylase 9.6 kb (msTH) and the mouse prion protein (msprp). In this study we compared the regional and cellular expression pattern of the transgenic protein in the brain and peripheral organs of various transgenic mouse lines. Western blot analysis and immunohistochemistry consistently showed that all three promoters successfully drive the expression of the transgene. The msprp promoter was found to give the highest level of transgene expression. All promoters directed the expression into the brain and specific neuron types. However, the promoters differed with respect to (i) the expression pattern in peripheral organs, (ii) the number and (iii) the regional distribution of expressing cells in the brain. Furthermore, remarkable line-to-line variation of expression patterns was observed in mouse lines carrying the same construct. Future studies will analyze how the variations in transgene expression affect the pathogenesis in the animals.

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Abbreviations

asyn	α-synuclein
ch	chicken
CMV-IE	cytomegalovirus enhancer
E	exon
GAD	glutamic acid decarboxylase
hm	human
hm^{2}asyn	human α-synuclein with two point mutations
LM	littermate
ms	mouse
PBS	phosphate-buffered saline
prp	prion protein
SA	splice acceptor
SD	splice donor
SV40	simian virus 40
TG	transgenic
TH	tyrosine hydroxylase

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Introduction

α-Synuclein (asyn), first isolated in 1988 from synaptic vesicles of *Torpedo californica* and rat brain [1], is a member of a protein family consisting of α-, β-, and γ-synuclein [2, 3]. It is a small 140-aa protein that is abundantly expressed in the brain and highly enriched in presynaptic vesicles [1, 4]. Linkage of the PARK1 locus on chromosome 4q21, where the asyn gene is located, to autosomal dominant Parkinson's disease (PD) in a large Greek family [5] suggested that asyn might be implicated in the pathogenesis of PD. Recent studies have supported this hypothesis, showing that (i) three missense mutations resulting in an Ala53Thr (A53T) [6], an Ala30Pro (A30P) [7] or an E64K [8] exchange, respectively, as well as a triplication of the entire wild-type gene [9] are associated with familial forms of PD, (ii) asyn protein is the most abundant component of Lewy bodies [10, 11] and (iii) a polymorphism in the 5′-untranslated region of the asyn gene correlates with an increased PD risk [12].

However, the question remains which role asyn plays in the pathogenesis of PD. Animal models based on the transgenic (TG) expression of wild-type and mutated genes provide powerful tools to elucidate the functional role of the encoded proteins. Hence, to date, several TG animals overexpressing either wild-type or mutant human asyn have been generated [13–24]. Unfortunately, the asyn transgenic lines (asynTG) exhibited different phenotypes and none of them replicated the complete PD pathology. This phenotypic inconsistency is likely caused by the different promoters used to drive transgene expression, by different integration sites and/or by different levels of transgene expression.

To elucidate the role of the promoter on the expression pattern of a transgene, we generated several synTG lines in which the expression of the doubly mutated human asyn (hm^2asyn) is driven by three different promoters. We used the chicken β-actin (chβactin) promoter, the 9.6-kb mouse tyrosine hydroxylase (msTH) promoter, and the mouse prion protein (msprp) promoter. In several synTG mouse lines from each promoter construct, we compared the regional and cellular expression of the transgene in the brain and peripheral organs.

Materials and Methods

Construction of hm^2asyn TG Animals

For the present study we generated TG mice expressing hm^2asyn under the control of three different promoters: the chβactin, the msTH, and the msprp promoter.

Human asyn cDNA was obtained by RT-PCR. The two PD-associated mutations were introduced by in vitro mutagenesis (Quickchange kit; Stratagene, Calif., USA). To maintain SV40 SD/SA and polyA elements, the mutated cDNA was ligated into the vector pCMV-β (Clontech, Calif., USA) after restriction with *Xho*I and removal of the overhanging ends by treatment with the Klenow fragment of DNA polymerase I. The cDNA with the adjacent SV40 elements was separated from the vector by restriction with *Xba*I.

For the overexpression with the chβactin promoter the hm^2asyn cDNA was cloned into the XhoI site of the vector pCAGGS (Gunma University School of Medicine, Japan). Due to the rb β-globin polyA and CMV-IE enhancer in this vector, the asyn cDNA was inserted without the SV40 elements (fig. 1).

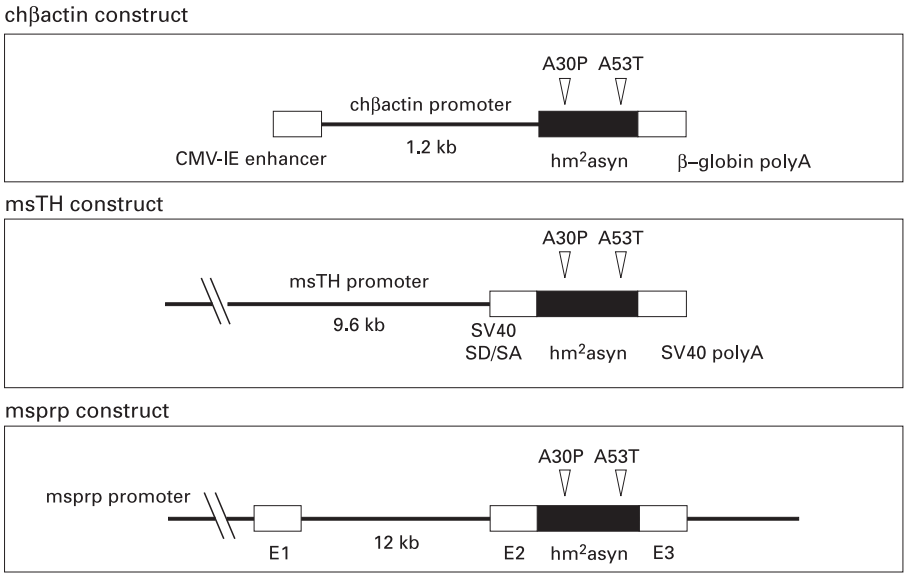

Fig. 1. Schematic representation of the chβactin, the msTH and the msprp construct. CMV-IE, cytomegalovirus enhancer promoter; E1–E3: exon 1–exon 3; SD/SA: splice donor/splice acceptor; SV40: Simian virus-40.

A 9.6-kb region of the msTH promoter (Genbank accession No. ACO12382) was cloned using several subclones of BAC clone 266D14 (restriction sites *Eco*RV and *XhoI*). The 815-bp fragment between the ATG and XhoI restriction site was amplified by PCR (primer THPRs: CCAGCTGAGAATGGGGGCTGCC, THPRas1: TCTAGAGCTCCTCCTCCCGAGTTCTG). The PCR fragment was analyzed for correct sequence and cloned into pBluescript KS(+) together with the promoter. The hm^{2}asyn with the SV40 elements was integrated by restriction with *Xba*I (fig. 1).

For the msprp construct, the hm^{2}asyn cDNA was inserted into vector msPrP.XhoI (Johns Hopkins University, Md., USA) by restriction with *Xho*I without the adjacent SV40 elements (fig. 1).

All constructs were sequenced and introduced into the germ line of C57 BL/6 mice by pronuclear injection of one-cell mouse embryos. Founders and offspring were identified by Southern blot and PCR analysis of tail DNA. TG mice were bred by crossing them with mouse strain C57BL/6. For the different constructs the following number of founders/stably expressing lines were generated: msprp 3/2, chβactin 10/3 and msTH 5/3. For all experiments, heterozygous TG and non-TG littermates (LM) were used. Animals were housed and maintained in accordance with the German guidelines of the animal care and use of laboratory animals.

Transfections

COS-1 cells (ATCC No. CRL-1650) cultured in growth medium, DMEM (Invitrogen, San Diego, Calif., USA), supplemented with 10% FBS (Invitrogen) in a humidified chamber with 8% CO_2, were seeded into 6-cm plates 1 day prior to transfection. Transfections with the constructs were performed by using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. The following day cells were seeded into 10-cm plates and grown until confluence for another 48 or 72 h. Cells were scraped off in Ca^{2+}-free phosphate-buffered saline (PBS; Invitrogen), collected by centrifugation, and washed twice in PBS. Pellets were frozen in liquid nitrogen and stored at –80°C until use.

Tissue Preparation

The TG animals as well as the non-TG LMs were divided into two groups. One group for Western blot analysis was sacrificed by decapitation. Brain regions were dissected and stored at –80°C until analysis. The other group was transcardially perfused with PBS followed by 4% paraformaldehyde (PA) in 0.1 *M* phosphate buffer.

For immunohistochemistry (ICC) perfusion-fixed brains were embedded in paraffin. Serial, frontal or sagittal, 5- or 18-μm-thick sections were cut throughout the brain for each mouse. All sections were mounted on Superfrost slides (Roth, Germany).

Protein Extraction and Immunoblots

For immunoblotting, dissected mouse brain regions were homogenized in homogenization buffer (0.32 *M* saccharose, 1 protease inhibitor tablet in 50 ml; Hoffmann-La Roche, Switzerland) containing complete protease inhibitor cocktail (Boehringer Mannheim, Germany) using Teflon/glass homogenizer at 4°C. Homogenized samples were left on ice for 30 min and centrifuged at 13,200 rpm (16,400 *g*) in a precooled centrifuge. The supernatant was diluted 1:1 in 2× Laemmli sample buffer and boiled. Protein determination was performed by the method of Neuhoff et al. [25].

Proteins were electrophoretically separated (15–30 μg protein/lane) on a 15% polyacrylamide gel-containing sodium dodecyl sulfate and transferred onto polyvinylidene difluoride membranes (Millipore, UK) at 4°C with 200 mA for 1.5 h. Blocking was performed with 3% milk powder and 2% bovine serum albumin in TBS-Tween (0.1% Tween, 20 m*M* TBS) at room temperature (RT) for 1 h. First, the membranes were incubated with an antibody against human asyn (Syn211, 1:1,000, diluted in blocking buffer; Zymed, Germany) overnight at 4°C, followed by washing in TBS-Tween and a second incubation with HRP-coupled secondary antibody (1:10,000; Amersham, Germany) in 1% milk powder, 0.5% bovine serum albumin in TBS-Tween at RT for 1.5 h. The blots were washed and developed using the ECL-Plus system (Amersham, Germany). After detection, the membranes were stripped with Restore stripping buffer (Pierce, Ill., USA) for 10 min at RT, incubated with a monoclonal antibody against β-actin (1:1,000; Sigma, Germany) and processed for detection as described above.

Films were digitized using a Cannon Power Shot Pro70 camera and analyzed using ImagePro software. Mean relative optical density in fixed measurement frames over the band of interest was corrected for background and protein content (corresponding β-actin band).

Immunohistochemistry

Deparaffinized and rehydrated sections were used for immunohistochemistry. Antigen retrieval (5-min cooking in 0.01 *M* citrate buffer pH 6.0) was performed with all sections. To reduce nonspecific binding, incubation sections were treated with 3% normal serum in 0.01 *M* PBS for 10 min by RT prior to antisera incubation.

Expression of hm^{2}asyn protein was analyzed with a monoclonal antibody specific for hmasyn (Syn211, 1:500). Following the first antiserum incubations, sections were treated by the corresponding biotinylated secondary antibody, ABC reagent and a silver-gold intensification [26]. Specificity of the stainings was confirmed by omitting primary antibodies.

For co-localization of hm^{2}asyn and the neurotransmitters dopamine and γ-aminobutyric acid, 5-μm-thick adjacent sections were either incubated with Syn211 or a sheep polyclonal antiserum against sheep tyrosine hydroxylase (TH, 1:500; Chemicon, Germany) and a monoclonal antibody against mouse glutamic acid decarboxylase (GAD, 1:5,000; Affinity, UK), respectively. Following, sections were processed as described above.

Data Analysis

Brain sections were visualized at the microscopic level (Axioscop 2; Zeiss, Germany) under brightfield illumination. Structures were described according to the main subdivisions of the brain and were identified with the aid of the atlas of Paxinos and Franklin [27]. Images were captured with an imaging system (Sony MC3255 camera) connected to a computer equipped with an image program (KS100 Rel.3.0). For final output, images were processed using Photoimpact 4 software.

Results

Three different promoters were tested for the expression of doubly mutated α-synuclein (hm^{2}asyn) in the mouse brain. Lines transmitting the transgene to offsprings were analyzed by Western blot analysis and immunohistochemistry for differences in regional and cellular expression patterns. No TG line showed loss of neu-

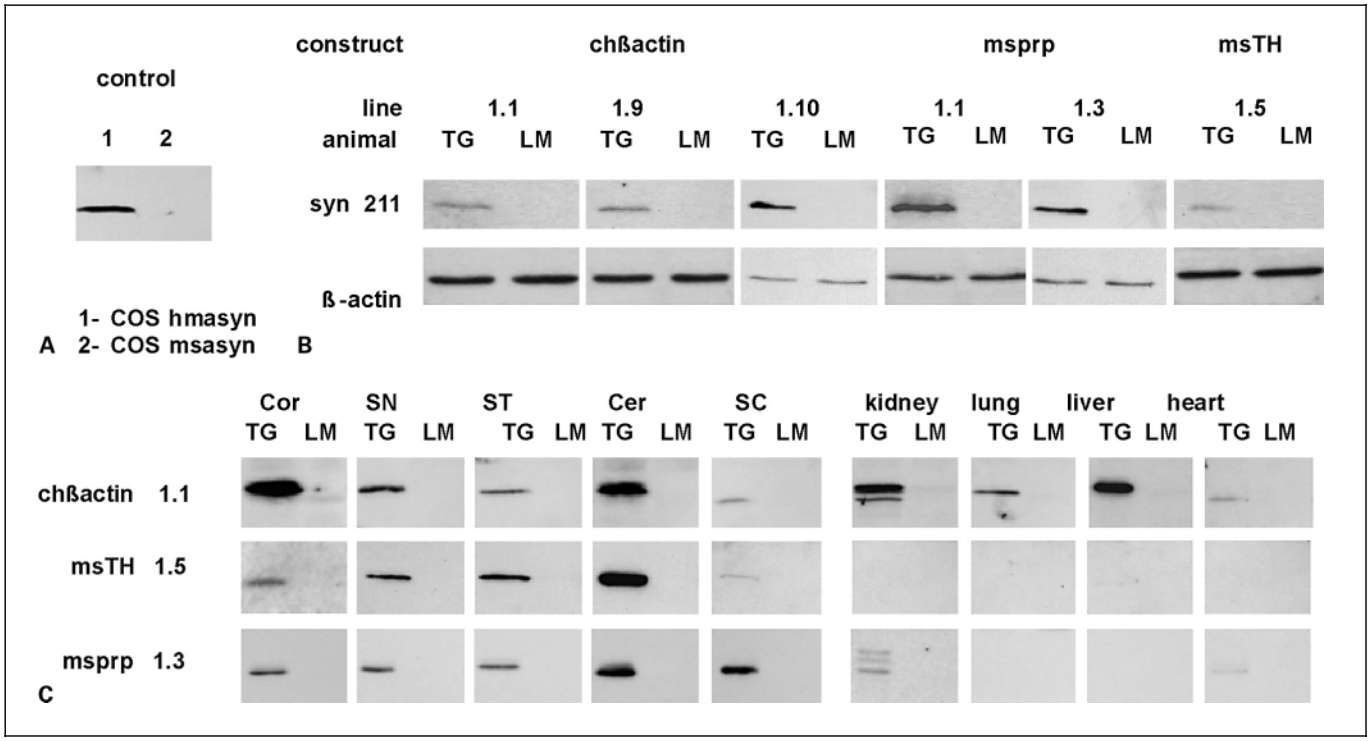

Fig. 2. Western blot analysis probed with human-specific asyn antibody (Syn211). **A** The Syn211-antibody is specific for human asyn, as shown by the strong band at about 16 kD in the COS cells transfected with human asyn (1) and the absence of labeling in the COS cells transfected with mouse asyn (2). **B**, **C** Protein extracts of total brain (**B**, 20 μg protein/lane), different brain regions (**C**, chβactin 1.1 and msTH 1.5–30 μg protein/lane; msprp 1.3–15 μg protein/lane) and peripheral organs (**C**, 20 μg protein/lane) of 2- to 4-month-old TG mice were incubated with Syn211. Blots shown in **B** were stripped and probed with an antibody against β-actin to check for equal loading. Note (i) the difference in expression levels in total brain, (ii) the absence of hm^2asyn expression in the kidney, the lung, the liver and the heart of the msTH-driven TGs, (iii) the kidney-specific appearance of an asyn fragment in the chβactin line 1.1 and (iv) the higher molecular weight forms of asyn displayed by the kidney of the msprp line 1.3. Cer = Cerebellum; Cor = cortex; SC = spinal cord; SN = substantia nigra; ST = striatum.

rons in the substantia nigra compacta. However, the msTH lines developed mitochondrial changes at the age of 2 months (data not shown).

Localization of the TG Protein in Mouse Tissues

Using Western blot analysis, we analyzed the expression levels and regional distribution patterns of the transgene in different organs of one line for each construct. To specifically detect hm^2asyn we used the monoclonal antibody Syn211. Western blot analysis of COS-1 cells transfected with either human or mouse asyn (fig. 2A) and immunohistochemistry of wild-type mouse tissue (fig. 4F, and see below) demonstrated the specificity of Syn211 for human asyn with no cross-reactivity to mouse asyn.

All TG lines expressed the hm^2asyn in the brain and only the chβactin and the msprp lines showed expression in peripheral organs.

In total brain, lines with the msprp construct showed the highest level of expression (fig. 2B), about four times higher than that induced by the chβactin and msTH promoters.

All constructs induced transgene expression in several brain regions (fig. 2C). The level of expression varied among the different brain regions, but was always strongest in the cerebellum.

With respect to peripheral organs, animals with the chβactin construct displayed a strong band in the kidney and the liver, while lung and heart displayed only moderate levels of the transgene (fig. 2C). The msprp construct induced some faint expression in the kidney and the heart, while in the msTH animals the transgene expression was restricted to the brain and spinal cord (fig. 2C).

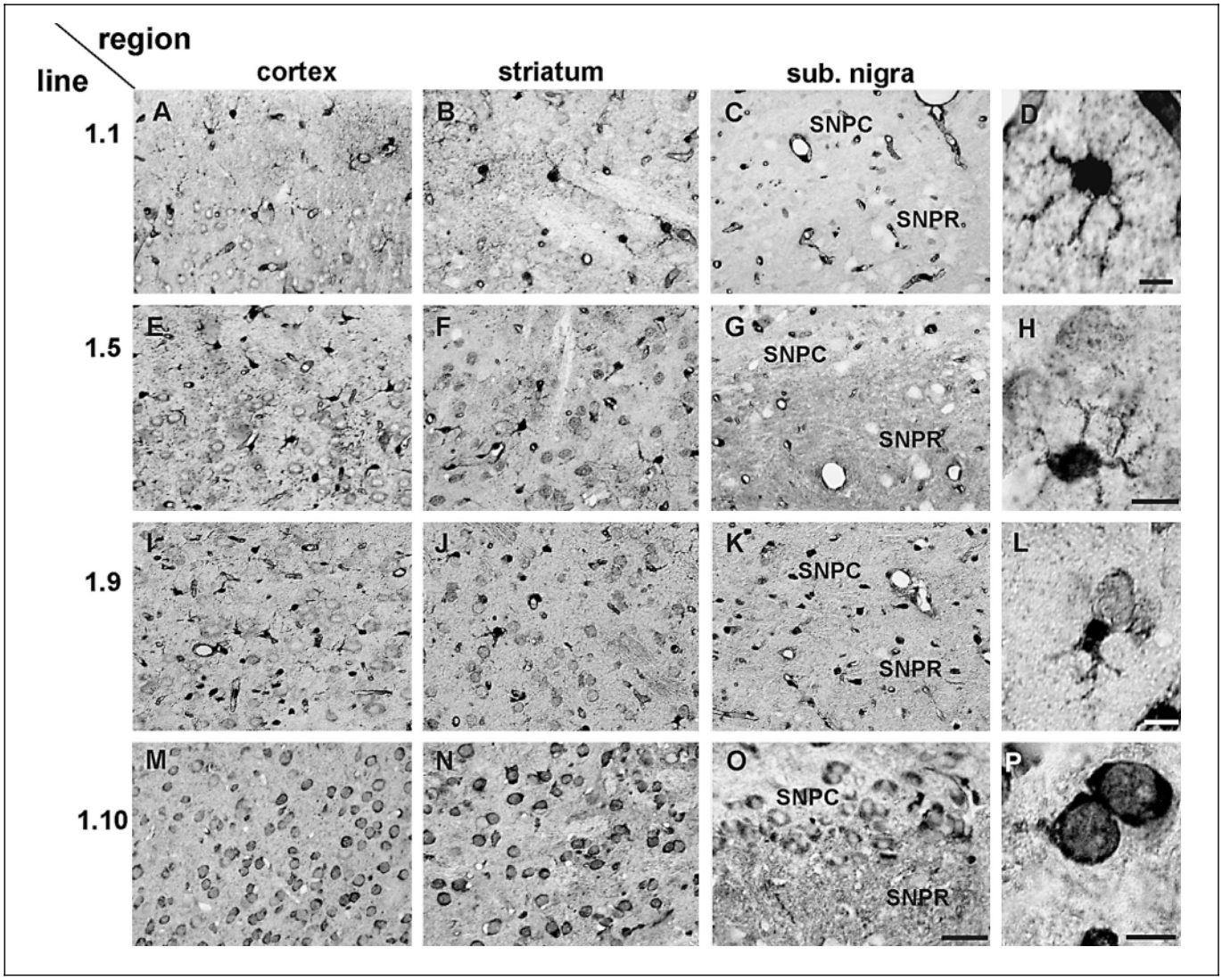

Fig. 3. chβactin lines. Expression of hm^{2}asyn protein in chβactin line 1.1 (**A**–**D**), line 1.5 (**E**–**H**), line 1.9 (**I**–**L**) and line 1.10 (**M**–**P**) at the age of 4–6 weeks. All lines expressing the hm^{2}asyn protein under the control of chβactin promoter exhibit numerous immunopositive cells in the cortex, the striatum and the substantia nigra. While lines 1.1, 1.5 and 1.9 show neuronal as well as glial labeling (**D**, **H**, **L**), only neurons were stained in the line 1.10 (**P**). Note that the latter also displays staining of the dopaminergic neurons in the substantia nigra pars compacta. SNPC = Substantia nigra pars compacta; SNPR = substantia nigra pars reticulata. Scale bars, 250 μm (**O**, also for **A**–**C**, **E**–**G**, **I**–**K**, **M**, **N**); 100 μm (**D**, **L**); 50 μm (**H**, **P**).

Transgene Expression Pattern in the Brain

The patterns of hmasyn protein in the brain were investigated in four chβactin-hm^{2}asyn, two msTH-hm^{2}asyn and two msprp-hm^{2}asyn lines. All lines showed transgene expression throughout the brain, but both the regional expression level and the cells expressing the transgene varied among the different constructs. Moreover, we also observed that lines carrying the same construct exhibit differences in expression patterns.

Regional Expression Pattern

While the chβactin promoter induced high levels of hm^{2}asyn in all brain divisions, the regional expression patterns of the four lines differed considerably from one another (fig. 3-5; table 1). In contrast to line 1.10, which exhibited strong expression in almost all brain regions analyzed, lines 1.1, 1.5 and 1.9 showed much lower expression levels (fig. 3, table 1). The most remarkable difference was observed in the nigrostriatal system. Only

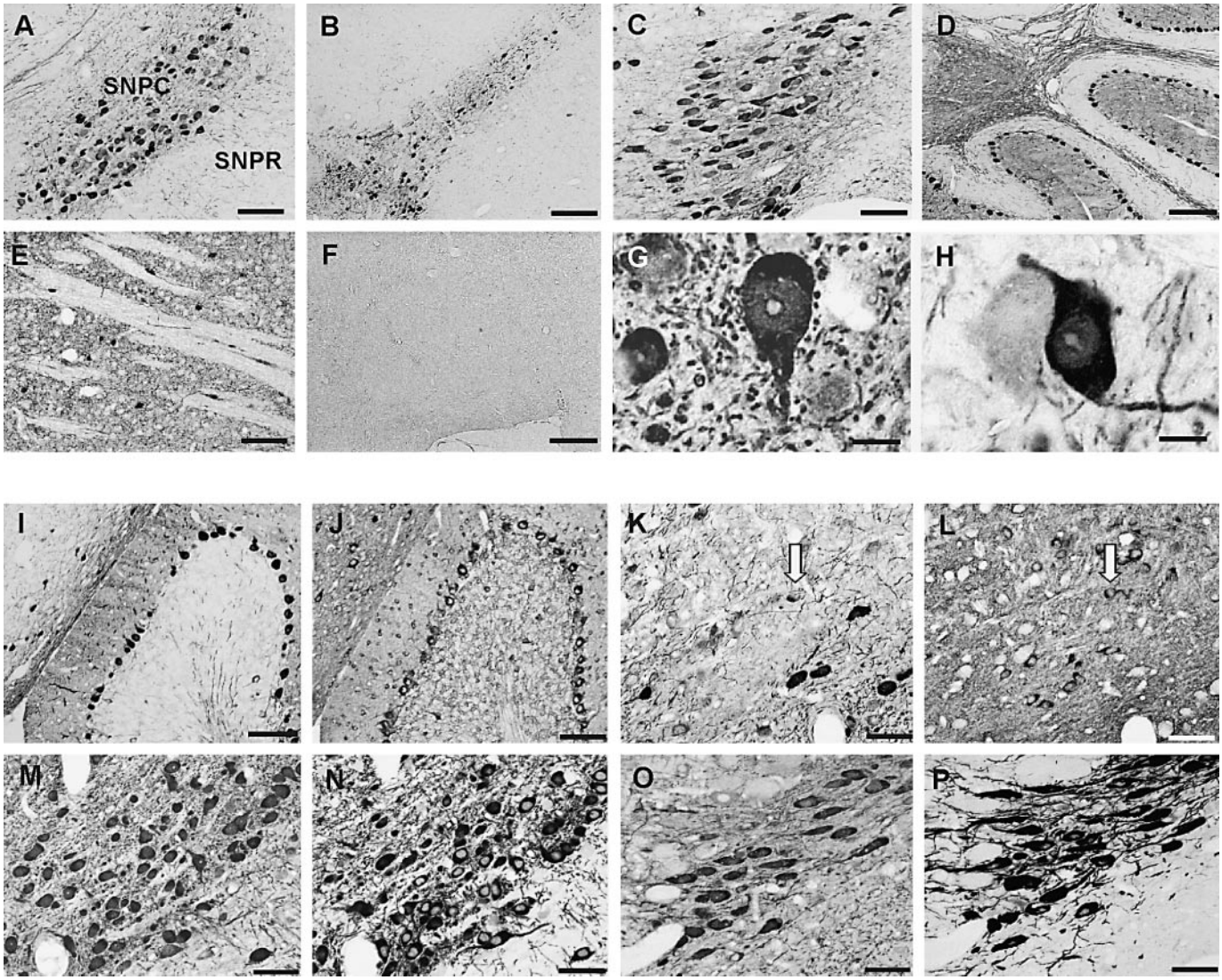

Fig. 4. msTH line 1.5. **A**–**H** Expression of hm^{2}asyn protein in 6-week-old animals of msTH line 1.5. Strong immunostained neurons distributed in the substantia nigra (**A**), the retrorubral field (**B**), the locus coeruleus (**C**), the cerebellum (**D**) and the striatum (**E**). **F** Non-TG littermate is free of immunoreactivity. **G**, **H** High magnification of neurons expressing hm^{2}asyn protein. **I**–**P** Staining of adjacent 5-μm-thick sections with Syn211 (**I**, **K**, **M**, **O**) and GAD (**J**, **L**) or TH (**N**, **P**). In the cerebellum (**I**, **J**) all Purkinje cells and various interneurons, and in the substantia nigra pars reticulata (**K**, **L**) single (marked by an arrow) gabaergic neurons express the hm^{2}asyn protein. Transgene expression is mostly found in catecholaminergic neurons, as shown here in the substantia nigra pars compacta (**M**, **N**) and the locus coeruleus (**O**, **P**). Scale bars, 500 μm (**A**, **C**, **E**, **I**, **J**); 1 mm (**B**, **D**, **F**); 50 μm (**G**, **H**); 250 μm (**K**–**P**).

one of four lines (1.10) exhibited strong expression in the substantia nigra and the striatum (fig. 3, table 1). Moreover, the most abundant expression within this line was seen in the cerebral cortex, the hippocampus and the thalamus.

In contrast to the latter, the msTH promoter induced a more distinct expression pattern. The number of hm^{2}asyn-positive cells was much lower and the distribution of expressing cells mirrored exactly the endogenous TH gene expression in the adult mouse and through development (fig. 4A–H, table 1). The latter relates to the fact that animals exhibited ectopic expression at several sites that express TH in the embryo but do not maintain expression in the adult such as the cerebellum (fig. 4D) and the inferior colliculus. In contrast to the chβactin lines, the expression patterns were almost identical in the

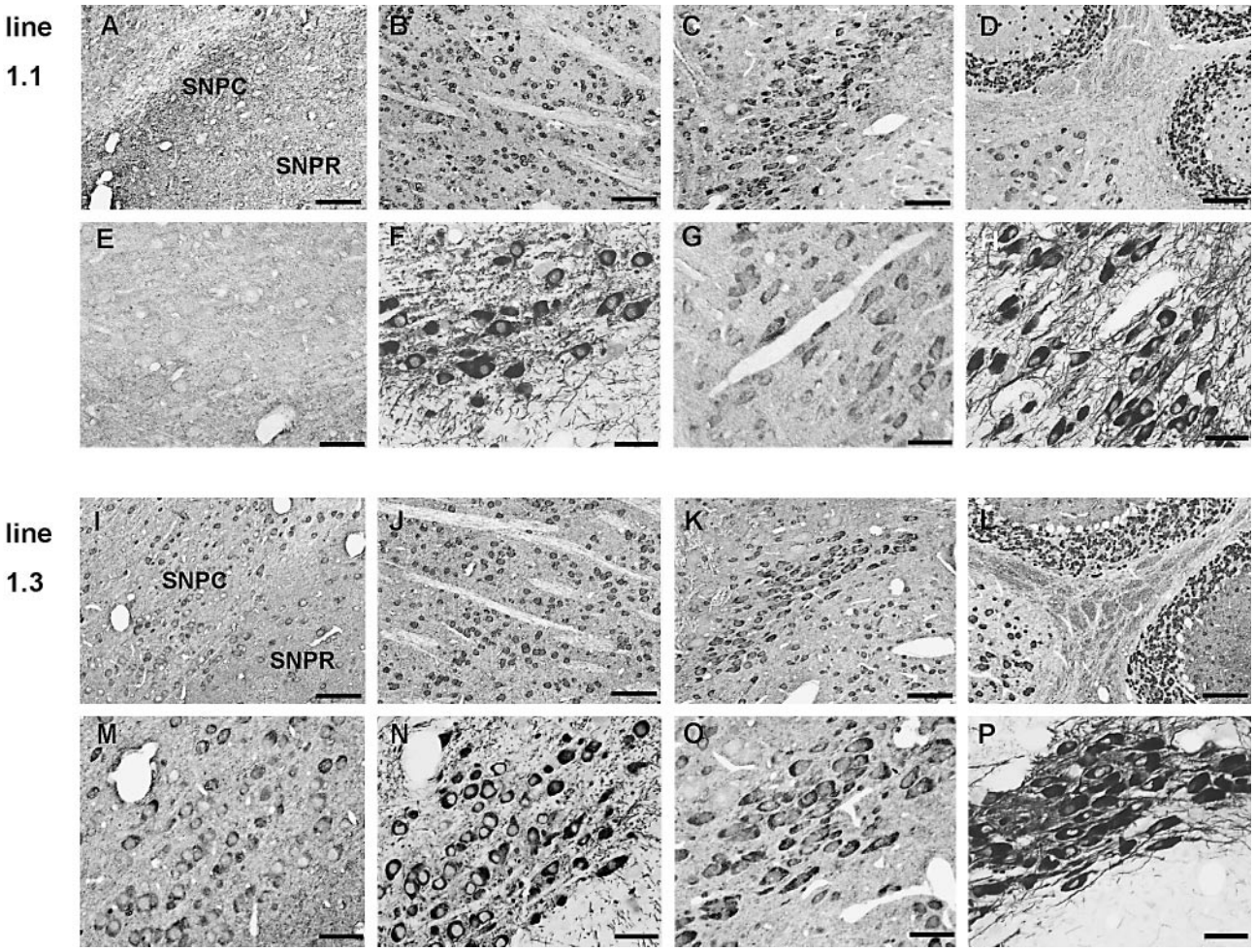

Fig. 5. msprp lines. **A**–**D**, **I**–**L** Expression of hm^{2}asyn protein in 4- to 8-week-old mice of lines 1.1 (**A**–**D**) and 1.3 (**I**–**L**). Both lines show numerous expressing neurons in the striatum (**B**, **J**), the locus coeruleus (**C**, **K**) and the cerebellum (**D**, **L**). In contrast to the substantia nigra of line 1.1 which is free of immunopositive cells (**A**, **E**), that of line 1.3 exhibits hm^{2}asyn protein-expressing neurons. **E**–**H**, **M**–**P** Staining of adjacent 5-μm-thick paraffin sections with Syn211 (**E**, **G**, **M**, **O**) or TH (**F**, **H**, **N**, **P**) demonstrate the expression of hm^{2}asyn protein in catecholaminergic neurons of the substantia nigra in line 1.3 and of the locus coeruleus in lines 1.1 and 1.3. Note, that the dopaminergic neurons in the substantia nigra of line 1.1 do not express the transgene. Scale bars, 500 μm (**A**–**D**, **I**–**L**); 250 μm (**E**–**H**, **M**–**P**).

two msTH lines analyzed and differed only in few regions (table 1). In general, the highest expression levels were found in the mesencephalon, while the diencephalon was devoid of hm^{2}asyn-expressing cells.

The msprp construct favored a strong expression in all brain regions. However, similar to the chβactin lines, mice carrying the msprp promoter differed with respect to mesencephalic expression (fig. 5, table 1). While one line (1.3) showed strong, neuronal hm^{2}asyn staining in the substantia nigra (fig. 5I), the other (1.1) exhibited no neuronal staining in this region (fig. 5A). Major expression sites were the cerebral cortex, the hippocampus, the colliculi and the granular layer of the cerebellar cortex.

Cellular Expression Pattern

Two of the promoters used, the msTH and the msprp, targeted the transgene exclusively to neurons (fig. 4, 5). In contrast to these, the cellular pattern in chβactin lines varied from an exclusive neuronal to a more general mixed glial-neuronal pattern. In particular, three of the chβactin lines (1.1, 1.5, 1.9) displayed numerous hm^{2}asyn-expressing glial cells throughout the brain (fig. 3D, H, L).

Table 1. Expression pattern of hm^{2}asyn in different synTG lines

Construct:	chβactin							msTH		msprp	
Transgenic line:	1.1		1.5		1.9		1.10	1.2	1.5	1.1	1.3
Cell types:	neurons	glia	neurons	glia	neurons	glia	neurons	neurons	neurons	neurons	neurons
Telencephalon											
Olfactory bulb	–	(+)	+	+	(+)	+	7	++	++	+	+
Striatum	–	+	+	+	+	+	++	(+)	+	+	+
Lateral globus pallidus	/	/	/	/	(+)	+	+	–	(+)	–	(+)
Nucleus accumbens	–	+	+	+	+	+	++	+	+	+	+
Hippocampus (CA1)											
Molecular layer	–	+	–	+	(+)	(+)	(+)	–	–	(+)	(+)
Pyramidal layer	+	–	+	–	++	–	++	–	–	++	++
Multiform layer	–	+	–	+	(+)	(+)	++	–	–	(+)	(+)
Hippocampus (CA2)											
Molecular layer	–	(+)	–	(+)	(+)	(+)	(+)	–	–	(+)	(+)
Pyramidal layer	(+)	–	–	–	++	–	++	–	–	+	+
Multiform layer	–	(+)	–	(+)	(+)	(+)	++	–	–	(+)	(+)
Hippocampus (CA3)											
Molecular layer	–	(+)	–	(+)	(+)	(+)	(+)	–	–	(+)	(+)
Pyramidal layer	(+)	–	–	–	+	–	++	–	–	+	++
Multiform layer	–	(+)	–	(+)	(+)	(+)	++	–		+	++
Dentate gyrus											
Molecular layer	–	++	–	++	(+)	(+)	(+)	–	–	–	–
Granular layer	(+)	+	–	(+)	++	–	++	–	–	++	++
Multiform layer	–	(+)	–	(+)	(+)	(+)	+	–	–	(+)	(+)
Subiculum	–	+	–	++	+	+	+	–	+	+	++
Corpus callosum	–	++	–	++	–	++	–	–	–	–	–
Motor cortex	(+)	+	(+)	+	+	+	++	(+)	+	++	++
Layers	1–6	1–6	1–6	1–6	1–6	1–6	1–6	3–6	3–6	1–6	1–6
Visual cortex	(+)	++	(+)	+	+	+	++	(+)	+	++	++
Layers	1–6	1–6	1–6	1–6	1–6	1–6	1–6	3–6	3–6	1–6	1–6
Entorhinal cortex	(+)	+	–	+	–	+	++ +	/	(+)	++	++
Layers	1–6	1–6	1–6	1–6	1–6	1–6	1–3 4–6	/	5+6	1–6	1–6
Nucleus amygdaloideus	–	(+)	–	+	–	+	+	(+)	+	(+)	+
Medial septal nucleus	–	(+)	/	/	–	+	+	–	+	+	++
Diencephalon											
Thalmus	–	++	–	+	(+)	++	++	–	–	+	+
Hypothalmus	(+)	(+)	–	+	(+)	++	+	–	(+)	+	+
Zona incerta	/	/	–	+	–	+	+	–	–	+	+
Subthalmic nucleus	/	/	–	+	–	+	+	–	–	+	+
Mesencephalon											
Inferior colliculus	–	+	(+)	++	(+)	++	/	+	+	++	++
Superior colliculus	(+)	(+)	–	++	(+)	++	+	(+)	(+)	++	++
Retrorubral field	–	(+)	–	+	–	+	+	+	+	+	+
Substantia nigra pars comp.	–	(+)	–	+	–	+	+	++	++	–	+
Substantia nigra pars reticul.	–	(+)	–	(+)	–	+	–	(1)	(1)	–	+
Ventral tegmental area	–	(+)	–	+	–	+	+	++	++	–	+
Metencephalon											
Locus coeruleus	–	–	–	+	+		+	+	+	+	+
Nuclei pontis	–	+	–	+	–		–	–	–	+	+
Cerebellum											
Molecular layer	–	–	–	(+)	–		–	+	+	+	+
Purkinje cell layer	+	–	+	–	++		+	++	++	–	–
Granular layer	+	–	–	+	–		+	(+)	(+)	++	++
White matter	–	–	–	+	–		+	–	–	–	–
Cerebellar nuclei	–	–	(+)	(+)	–		–	++	++	+	+
Myelencephalon											
Motor trigeminal nuclei (V)	–	–	–	+	–		+	–	–	–	–
Facial nucleus (VII)	+	–	–	+	–		+	–	–	+	+
Nuclei formatio reticularis	(+)	(+)	(+)	++	(+)		++	–	–	+	+
Raphe magnus nucleus	/	/	–	+	–		/	/	/	/	+

To characterize the transgene-expressing neurons further, we stained adjacent sections with sgk1 or antibodies against different neurotransmitter enzymes, respectively. With the exception of some ectopic expression sites, e.g. the GAD-immunopositive Purkinje cells (fig. 4I, J) and individual cells in other brain regions, almost all neurons in the msTH lines displayed the dopaminergic marker TH (fig. 4M–P). Besides the msTH lines, only one chβactin-(1.10; fig. 3O) as well as one msprp-line (1.3; fig. 5G, H, M–P) showed co-localization of TH and the transgene hm^{2}asyn in the nigrostriatal system.

Cellular expression of the hm^{2}asyn protein was strong in all TG lines. The hm^{2}asyn reaction product filled the somata and the processes of expressing cells. In all TG lines hm^{2}asyn protein was also found in the nucleus of some cells, but always spared the nucleolus (fig. 3P).

Discussion

To address the influence of promoters on the neuronal expression pattern of a transgene, we created multiple lines of TG mice expressing the doubly mutated human asyn under the control of three different promoters: the chβactin, the msTH, and the msprp promoter. In this study we have provided a detailed description of the regional distribution and the nature of the transgene-expressing cells in the brain.

Our results demonstrate that all promoters used (i) effectively induce high transgene expression, (ii) direct the expression to the brain, and (iii) target neurons. However, the constructs used varied with respect to (i) the expression in peripheral organs, and (ii) the number and (iii) the regional distribution of expressing cells in the brain.

Western blot analysis and immunohistochemistry consistently showed that all three promoters successfully drive the expression of the transgene hm^{2}asyn. While the expression in the peripheral organs was variable and depended on the promoter chosen, a strong expression in the brain was constantly present in all transgenes. The widespread and strong expression of chβactin-driven transgenes is a well-known phenomenon and has been described in several reports [28–31]. Studies of the expression of endogenous prp in adult and developing mice [32, 33] and the expression pattern of a prp-driven transgene [34] implicate a peripheral expression of the promoter, as observed in this study. However, the results of the latter publication differed slightly from our peripheral pattern, which had strong expression in the heart, but none in the kidney. No expression was seen in the kidney, the lung, the liver and the heart using the msTH promoter.

Two of the promoters, chβactin and msprp, targeted almost all neurons in the brain. With the exception of few regions, hm^{2}syn-expressing cells were distributed throughout the entire brain. Our data are consistent with previous reports of β-actin and prp distribution in non-TG and TG mice [15, 34–36], even though they are less detailed. In contrast to chβactin and msprp, the 9.6-kb msTH cassette directed the transgene specifically to a subpopulation of neurons; the catecholaminergic neurons plus some neurons that express TH transiently during development. The observed ectopic expression is a well-known phenomenon found in several transgenes with the rat TH promoter of varying length (4.5 kb up to 9 kb) [17–19, 37–39]. Since we did not succeed in repressing an ectopic expression by the use of an even longer 9.6-kb promoter from mouse, we conclude that our promoter likewise lacks a negative element that switches off TH expression in certain cells in adulthood.

Two of the promoters, the msTH and the msprp, directed the transgene exclusively into neurons, while the chβactin promoter also induced the expression of the transgene in glial cells. The neuron-specific distribution of the prp-driven transgene is surprising, since prp is also considered to be a glial protein [41, 42]. However, expression patterns of prp-driven transgenes are heterogeneous. While a prp-driven APP TG line exhibited astroglial expression in addition to robust neuronal expression [34], an asyn TG line exhibited an exclusive neuronal expression pattern [23]. Another remarkable and very important observation of the present study is the line-to-line variability of expression patterns in different mouse lines carrying the same construct. Every line exhibited a unique pattern of TG expression. This phenomenon was particularly prominent for the chβactin lines, where even different cell types were affected. Extremely variable patterns have before been reported for a chβactin-driven neomycin resistance gene [43], but also for other promoters like Thy-1 [44] and prp [23]. Variability of gene expression between TG lines is due to copy number and integration site. The integration site often has profound effects, ranging from silencing to enhancement of transgene expression. Our results further indicate that the chβactin promoter is more prone to line-to-line variations than the other promoters used in this study. Conversely, the msTH 9.6 kb promoter induced almost identical expression patterns in different mouse lines. However, analyses of more lines are necessary to confirm this assumption. Moreover,

because of the diversity in the integration sites, it might be expected that line-to-line variation may also result from unexpected changes in the transcription rate of endogenous genes at the integration site. All these experimental variations, arising from differences in the integration site and the number of integrated cassettes, should be considered when phenotypes of different lines with the same TG construct are compared.

Over the past years several asyn TG lines have been generated to study the involvement of asyn in PD pathogenesis and to make high-fidelity animal models resembling PD pathology. Some of them also used the promoters msprp [13, 15, 23] and msTH [17–19, 24] analyzed in this study, while a TG line using the β-actin promoter has not been reported before. Though some lines displayed at least some of the features of PD, animals with the same targeting vector from different groups generated inconsistent results. One such example are prp-driven asyn TG lines, which showed differences in neuronal pathology and in the time course of motor deficits [13, 15, 23]. This inconsistency, which might be caused by the integration process discussed above, is a major drawback of the TG approach. Targeting a TG sequence to a chosen location, e.g. by homologous recombination, may allow a more precise modelling of human disease.

In conclusion, TG animals offer a valuable approach to analyze pathogenesis of human diseases, and asyn transgenes driven by the TH promoter might be the most suitable models for the generation of PD pathology. However, when using the standard TG technique, one has to be aware that the results are not controlled and might be accompanied by unforeseen consequences.

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