Production of a monoclonal antibody, against human α-synuclein, in a subpopulation of C57BL/6J mice, presenting a deletion of the α-synuclein locus

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A T I C L E  I N  F O

Article history:
Received 13 July 2010
Received in revised form 5 August 2010
Accepted 6 August 2010

Keywords:
- α-Synuclein
- Antibody
- Immunization
- Lewy bodies
- Parkinson's disease

A B S T R A C T

Analyses using antibodies directed against α-synuclein play a key role in the understanding of the pathologies associated with neurodegenerative disorders such as Parkinson’s disease (PD), dementia with Lewy bodies (DLB) and multiple system atrophy (MSA). However, the generation of antibodies against immunogens with significant sequence similarity to host proteins such as α-synuclein is often hindered by host immunotolerance.

In contrast to wild-type C57BL/6j and BALB/c mice immunized with recombinant human α-synuclein, C57BL/6S Δsnca mice presenting a natural deletion of the α-synuclein locus, bypassed the immunotolerance process which resulted in a much higher polyclonal antibody response. The native or fibrillized conformation of α-synuclein used as the immunogen did not have an impact on the amount of specific antibodies in sera of the host. The immunization protocols resulted in the generation of the IgG AS11, raised against fibrillized recombinant human α-synuclein in C57BL/6S Δsnca mice. This monoclonal antibody, recognizing an N-terminal α-synuclein epitope, was selected for its specificity and significant reactivity in Western-blot, immunofluorescence and immunohistochemistry assays. The ability of AS11 to detect both soluble and aggregated forms of α-synuclein present in pathological cytoplasmic inclusions was further assessed using analysis of human brains with PD or MSA, transgenic mouse lines expressing A53T human α-synuclein, and cellular models expressing human α-synuclein.

Taken together, our study indicates that novel antibodies helpful to characterize alterations of α-synuclein leading to neurodegeneration in PD and related disorders could be efficiently developed using this original immunization strategy.

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1. Introduction

α-Synuclein is a 140 amino-acid neuronal protein predominantly present in synaptic junctions (Kahle et al., 2000). Although primarily a natively unfolded protein soluble in the cytosol (Weinreb et al., 1996), α-synuclein is also able to bind vesicular membranes by forming amphipathic α-helices, supporting its probable physiological role in the regulation of neurotransmitter release. However, α-synuclein is more specially studied for its relevance in understanding sporadic and familial forms of Parkinson’s disease (PD) and related neurodegenerative disorders. In fact, point mutations and copy number variations in the gene encoding α-synuclein (SNCA) are associated with inherited forms of PD, thereby establishing a link between alteration and accumulation of this protein and the development of neuropathologies (Polymeropoulos et al., 1997; Singleton et al., 2003). Moreover, α-synuclein has been identified as the major constituent of pathological intra-neuronal Lewy body inclusions (Spillantini et al., 1997), which are characteristic of PD and dementia with Lewy bodies (DLB). It is also the main component of the glial cytoplasmic inclusions present in multiple system atrophy (MSA) (Wakabayashi et al., 1998). The strong α-synuclein-associated immunoreactivity of these abnormal intra-neuronal or glial filaments, combined with a substantial loss of neurons is characteristic of synucleinopathies. Thus, antibodies having the ability to detect abnormal α-synuclein inclusions play a
key role in the understanding of pathological molecular processes leading to neurodegeneration. The production and characterization of several monoclonal antibodies directed against α-synuclein have been previously reported, and have provided useful information about their specificity, sensitivity, and the epitopes recognized (Baba et al., 1998; Choi et al., 2006; Croisier et al., 2006; Duda et al., 2002; Glasson et al., 2000b; Jakes et al., 1999; Perrin et al., 2003; Waxman et al., 2008; Yu et al., 2007). However, producing monoclonal antibodies (mAb) against an endogenous protein such as α-synuclein is often hindered by immunotolerance of the immunized animals, particularly since the amino acid sequence is highly conserved between species. In this study, we have tried to break the immunotolerance process by immunizing a subpopulation of C57BL/6J inbred mice presenting a natural deletion for \( \text{snca} \) (Specht and Schoepfer, 2001) with recombinant human α-synuclein.

2. Materials and methods

2.1. Ethics statement

Animal studies described in this report were carried out in our approved experimental facilities (n = A 69 387 081), in accordance with the EC Directive 86/609/EEC for animal experiments and with the approval of the CREEA (Regional Ethical Committee for Animal Experiments).

Human brain samples have been collected in the frame of a prospective community-based cohort study, the Vienna Trans-Danube Aging (VITA) Study, Vienna, Austria. Neuropathological studies within this project have full approval by the responsible ethics committee of the Medical University Vienna/Vienna General Hospital. VITA study participants had to sign an approved informed consent.

2.2. Immunization

In an attempt to promote different presentations of the immunogen, recombinant human α-synuclein (Sigma) (α-syn rec) was pretreated before injection. To promote oligomerized forms of α-synuclein, α-syn rec was subjected to high-salt or acid treatment.

(i) α-Synuclein modified under high-salt conditions (α-syn salt) was obtained by incubating α-syn rec in 1 M NaCl 25 mM Tris–HCl pH 7.5 at 37 °C for 1 week (Emadi et al., 2007; Yagi et al., 2005).

(ii) The modification of α-synuclein under acidic conditions (α-syn acid) was performed by incubating α-syn rec in 20 mM acetic buffer pH 3.5, for 24 h at 37 °C (Uversky et al., 2001).

During the first immunization protocol, 3 groups of 5 C57BL/6S \( \Delta \text{snca} \) (Harlan) presenting a natural deletion for \( \text{snca} \) were immunized with 1 μg of either α-syn rec, α-syn salt or α-syn acid by intra-peritoneal injection. Immunogens were diluted in complete Freund’s adjuvant. Twenty-one days later, the second immunization was performed with 1 μg of immunogen in incomplete Freund’s adjuvant. The third immunogen administration was given in PBS, 42 days after the beginning of the protocol. The final injection was made with 5 μg in the orbital sinus of mice at day 55. As controls, the same protocol was applied to three groups of five wild-type C57BL/6J mice (Charles River Laboratories).

To estimate the amount of polyclonal antibodies in the sera of immunized mice, blood samples were collected at days 0, 35 and 56. The quantification was achieved by indirect Enzyme-Linked Immunosorbent Assay (ELISA) using 96-wells microtiter plates (Maxisorp, Nunc) coated with 1 μg/ml of immunogen (either α-syn rec, or α-syn salt or acid). Sera were diluted in PBS 0.05% Tween 20 and incubated 1 h with shaking, followed by incubation with a HRP-conjugated goat anti-mouse IgG (H+L) antibody (P.A.R.I.S.) 1:1000. OPD (Sigma) was used as substrate and plates were read at wavelength 450 nm.

Subsequent to this first protocol of immunization, a second protocol including a longer schedule of immunization was performed on three different mouse lines: 4 C57BL/6J, 4 C57BL/6S \( \Delta \text{snca} \) and 4 BALB/c. The protocol was performed as previously described, with the following exceptions: injections were made at days 0, 14, 38 and 74. Blood samples were collected at days 0, 29 and 50 to estimate the amounts of circulating antibodies in an indirect ELISA. For this assay, a heavy chain specific anti-mouse IgG (Sigma) and a μ chain specific anti-mouse IgM (P.A.R.I.S.) were used.

2.3. Antibody generation

Three C57BL/6S (2 from the first protocol of immunization and 1 from the second) presenting the highest quantities of circulating anti-α-synuclein antibodies were selected for the production of monoclonal antibodies. Mice were euthanized 3 days after the last boost by administration of a lethal dose of sodium pentobarbital. Spleens were taken for obtaining splenocytes and fusion with SP2/0 myeloma cells was performed in presence of polyethylene glycol. Hybridomas were grown in DMEM-F12-Glutamax I medium (Invitrogen) supplemented with 10% horse serum ( Gibco), 0.1 mM hypoxanthine and 0.5% azaserine in 96-well plates. Hybridomas were screened for the production of anti-α-synuclein antibodies in an indirect ELISA assay on microplates coated with the immunogen. The assay was performed as previously described for the estimation of polyclonal antibody response, with the following exceptions: cell culture supernatants were tested undiluted and TMB was used as a substrate for HRP. The reaction was stopped with HCl 1 N and optical densities were read at 450 nm.

Cloning of positive hybridomas was performed by limiting dilution in 96-well plates. Isotyping of antibodies secreted in cell supernatants was performed by ELISA (Mouse mono Ab-kit(HRP)-Zymed Laboratories Inc.).

To produce large quantities of monoclonal anti-α-synuclein antibody AS11, ascites fluid were produced in BALB/c mice (Charles River Laboratories) treated with pristane (Sigma). Ascites fluid were purified on Affi-gel protein A agarose (BioRad) chromatographic columns. The concentration of purified anti-AS11 antibody was determined by Folin’s method (Lowry et al., 1951). Antibody AS11 was conjugated to HRP with the EZ-Link® Plus Activated Peroxydase kit (Pierce).

2.4. Gel electrophoresis and Western-blotting

Immunoreactivity of mAb AS11 was tested in Western-blot for the detection of full-length recombinant N-terminal histidine tagged human α-synuclein (Sigma) and peptides corresponding to (aa 61–140) and (aa 96–140) of human α-synuclein (r-Peptide). Brain protein extracts from C57BL/6S \( \Delta \text{snca} \), wild-type C57BL/6J, and the transgenic line M83 B6; C3H-Tg(SNCA)83Vle/J over-expressing A53T human α-synuclein (The Jackson Laboratory) (Glasson et al., 2002) were also tested. Brains from 3-month-old mice were homogenized in 3 ml/g of 50 mM Tris (pH 7.5), 750 mM NaCl, 5 mM EDTA and a cocktail of protease inhibitors (1%). Samples were centrifuged at 100,000 × g for 20 min. Supernatants were denatured in denaturing buffer (4% SDS, 2% β-mercaptoethanol, 192 mM glycine, 25 mM Tris, 5% sucrose) and heated 5 min at 100°C before being loaded on 15% gel for SDS-PAGE (Laemmli, 1970). Proteins were electroblotted onto PVDF membranes (Bio-Rad). Immuno detection was performed either directly with mAb AS11-HRP or with mAb 5C2 (SantaCruz Biotechnology) (1:5000 in PBST for 1 h) followed by incubation with a HRP-conjugated goat anti-mouse antibody (Pierce). The immunocomplexes were detected with chemiluminescent reagents (ECL Amersham), followed by exposure on Biomax MR Kodak films.
nants were amplified in analyzed by extensive restriction digest analysis. Positive recombi-
products of homologous recombination in BJ5183 cells were extracted by three freeze/thaw cycles and stored in PBS and 10%
glycerol. Viral titer was higher than 10⁸ plaque-forming units/ml.

Differentiation of neural progenitors was induced by the withdrawal of bFGF. NSCs were fixed at day 0 and day 6 of differentiation with 4%
paraformaldehyde (PFA).
The neuroblastoma cell line SH-SY5Y was maintained in DMEM-F12-Glutamax 1 medium containing 10% fetal bovine serum
(Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin in a
37 °C, 5% CO₂ incubator.

For SH-SY5Y transduction, a recombinant adeno-viral genome containing the full length cDNA encoding human WT α-synuclein in frame with a C-terminal myc-His epitope tag was generated by homologous recombination with 200 ng of SwaI-linearized VmAd-cDNA3.1 (a generous gift from S. Rusconi, Fribourg – Switzerland) and 600 ng of linearized pcDNA3.1 WT α-synuclein expression construct in chemically competent Escherichia coli XL-1 Blue cells, and purified DNA was
for one night at 4 °C. Antibodies were used at the following concentrations: anti-α-synuclein AS11 (1:200), anti-α-synuclein LB509 ((Abcam) 1:250), anti-nestin ((Millipore) 1:500), anti-β-III-tubulin
Tuj1 ((Eurogentec) 1:1000), anti-Glial Fibrillary Acidic Protein (GFAP) ((Dako) 1:1000) or anti-Bovine Immunodeficiency Virus
(BIV) p26 142 kindly provided by Susan Carpenter (Wannemuehler et al., 1993) (1:200). After washes with PBS-Tween 20 0.05%, cells were incubated with Alexa-Fluor conjugated goat secondary antibodies (Invitrogen) for 30 min. Nuclei were then stained with DAPI
((Sigma) 1:10,000 in PBS) for 5 min.

3. Results

3.1. Increased immunoreactivity in mice deleted for snca

During these immunization schedules, three different mouse lines (C57BL/6J, C57BL/6S Δsnca and BALB/c) were immunized with recombinant human α-synuclein. In order to compare their capacity to generate a humoral immune response against α-synuclein, the amounts of polyclonal anti-α-synuclein antibodies (pAb) secreted in their sera were followed throughout the immunization protocol. Negligible immune responses were observed in the sera of wild-type C57BL/6J mice during the first immunization protocol, as reflected by the very low amounts of total pAb detected using an anti-IgG (H + L) secondary antibody (Fig. 1A). This result was confirmed during the second immunization schedule, with the detection of low amounts of circulating IgG and IgM in sera of C57BL/6J, using an indirect ELISA with either an γ heavy chain specific anti-mouse IgG or an µ chain specific anti-mouse IgM (Fig. 2A).
In contrast, the majority of C57BL/6S Δsnca mice had medium or high levels of total circulating polyclonal anti-α-synuclein antibodies (Fig. 1B), consisting primarily of IgG, whereas IgM were moderately present (Fig. 2B). In sera of BALB/c, a mouse line commonly used in the production of monoclonal antibodies, levels of IgG were about two to three times lower than those obtained in C57BL/6S Δsnca mice (Fig. 2C). Given these results, the C57BL/6S mice lacking endogenous α-synuclein expression, seemed to be an efficient host to produce anti-α-synuclein antibodies.

Several conformational forms of recombinant human α-synuclein (α-syn salt, α-syn acid or untreated α-syn rec) were used as immunogens during the first immunization assay. The recombinant protein was treated following protocols known to generate oligomerized forms of α-synuclein (Emadi et al., 2007; Uversky et al., 2001; Yagi et al., 2005). The presence of higher molecular weight species of α-synuclein was verified by Western-blot (data not shown). The goal of these treatments was to modify the immunogen in order to increase the immunogenicity of α-synuclein. The ability of α-syn salt, α-syn acid or untreated α-syn rec to trigger the emergence of specific circulating antibodies in the sera of wild-type C57BL/6j or C57BL/6S Δsnca was determined by indirect ELISA. These assays demonstrated that the immunogenicity of α-synuclein was not modified by the treatment conditions, and the conformation of the immunogen had little impact on the strength of the immune response (Fig. 1).

3.2. Production of monoclonal antibody AS11, an IgG directed against human α-synuclein

Given the results from the study of the influence of the host and immunogen on the immune response, two C57BL/6S from the first immunization schedule presenting high levels of pAb were chosen for fusion. These mice were immunized with recombinant α-synuclein (α-syn rec), or α-synuclein aggregated at low pH (α-syn acid). Hybridomas splenocytes from the C57BL/6S immunized with α-syn rec, gave after cloning and screening in indirect ELISA using an anti-mouse IgG (H+L) secondary antibody, eight clones producing anti-α-synuclein antibodies. The second fusion done with the spleen of a C57BL/6S immunized with α-syn acid, allowed us to obtain 19 other clones producing mAb against α-synuclein. After their selection, each of these 27 monoclonal antibodies was used for hybridoma screening of the two first fusions, two different tests allowing the specific discrimination of IgG and IgM used for hybridoma screening of the two first fusions, two different tests allowing the specific discrimination of IgG and IgM were used to screen hybridomas from the third fusion. Contrary to the indirect ELISA with an anti-mouse IgG (H+L) secondary antibody used for hybridoma screening of the two first fusions, two different tests allowing the specific discrimination of IgG and IgM were used to screen hybridomas from the third fusion. This double screening assay led to the selection of 13 hybridomas producing IgM whereas no hybridoma producing IgG could be detected.
3.3. Detection of α-synuclein in Western-blot using HRP-conjugated AS11

Antibody AS11-HRP detected recombinant full-length human α-synuclein with an apparent molecular weight of ~20 kDa in Western-blot. Peptides (61–140 and 96–140) of α-synuclein were not detected by AS11, demonstrating that the N-terminal region of α-synuclein (1–60) was necessary for the recognition of AS11. As a control, full-length α-synuclein and peptides corresponding to (aa 61–140) and (aa 96–140) of human α-synuclein were detected by SC2, a human α-synuclein NAC (Non-Amyloid Component) domain specific IgG (epitope (aa 61–95)). As expected, antibody SC2 recognized both the full-length protein, and the peptide corresponding to (aa 61–140) of human α-synuclein, but failed to detect the C-terminal peptide (aa 96–140) (Fig. 3A).

Soluble human α3T5 α-synuclein extracted from brains of 3-month-old asymptomatic mice of transgenic line M83, could be detected in Western-blot by AS11-HRP. In contrast, no band corresponding to endogenous murine α-synuclein could be detected in C57BL/6J brain extracts at this dilution. Thus, AS11 was able to recognize recombinant wild-type or AS3T human α-synuclein, but failed to detect endogenous murine α-synuclein. Moreover, no band corresponding to α-synuclein was detected in cerebral extracts of α-synuclein deleted mouse. It should be noted however, that several non-specific bands of high molecular weight were present in all samples tested (Fig. 3B).

3.4. Specific detection of α-synuclein in neuronal cell-cultures by mAb AS11

Undifferentiated murine NSC derived from line M83 over-expressing human A53T α-synuclein were strongly detected by an antibody directed against nestin, a neuronal precursor specific protein (Fig. 4A). On the other hand, only weak immunostainings of β-III-tubulin (neuron specific) and GFAP (astrocyte specific), were observed at day 0 of differentiation (Fig. 4C). After 6 days of differentiation, cells were negative for nestin (Fig. 4B), consistent with the appearance of numerous neuronal and astrocytic cell types (Fig. 4D). Immunostaining of A53T human α-synuclein by antibody AS11 in NSCs derived from the transgenic line M83, was apparent at day 0 of differentiation (Fig. 4E). At day 6, mAb AS11 underlined the thin prolongations of neurons (Fig. 4F). In contrast, staining with mAb AS11 was absent at all stages of differentiation in cells derived from C57BL/6S Δsnca (Fig. 4G and 4H), thus demonstrating the specificity of this antibody.

AS11 could detect cytoplasmic α-synuclein in the human neuroblastoma cell line SH-SY5Y (Fig. 5A), and generated a stronger and finer staining than the commercially available anti-human α-synuclein mAb LB509 (Fig. 5B). The absence of immunostaining of SH-SY5Y cells with an irrelevant antibody (anti-BIV) was used as negative control (Fig. 5C). Moreover, strong immunofluorescence with mAb AS11 was observed in SH-SY5Y cells transduced with recombinant adeno-associated viruses designed to over-express human wild-type α-synuclein (rAAV α-syn WT) (Fig. 5D). Interestingly, AS11 was able to detect perinuclear inclusions containing aggregated α-synuclein in rAAV Δsyn WT transduced cells treated with 100 nM rotenone for 72 h (Fig. 5E). Similar aggresomes enriched in α-synuclein were detected with LB509 (Fig. 5F).

3.5. Immunohistochemical staining of pathological human brains by AS11

Intraneuronal inclusions (Lewy bodies) and Lewy neurites rich in fibrillized α-synuclein were accurately stained by AS11 in brainstem sections from patients with PD. Similarly, pathological glial cytoplasmic inclusions mainly found in oligodendrocytes in the pons of patients with MSA were specifically marked by AS11 (Fig. 6). The optimal working dilution was 1:500 with combined pretreatment of tissue with citrate buffer and formic acid prior to incubation with the primary antibody.

4. Discussion

The aim of this study was to evaluate the efficiency of several immunization pathways in the generation of monoclonal antibodies against α-synuclein. The production of several monoclonal α-synuclein antibodies has already been reported (Baba et al., 1998; Choi et al., 2006; Croisier et al., 2002; Gasson et al., 2000b; Jakes et al., 1999; Perrin et al., 2003; Waxman et al., 2008; Yu et al., 2007), but very few of these studies describe in detail the protocols and the hosts used for immunization. Nonetheless, these features deserve to be considered since the well conserved amino-acid sequence of α-synuclein increases the difficulty of producing antibodies in hosts expressing a similar endogenous protein. The primary amino acid sequences of human and murine α-synuclein share 95.3% homology (Lavedan, 1998), which likely hinders the recognition of the recombinant
human α-synuclein immunogen as a non-self protein. Similar difficulties in producing antibodies against the prion protein have been previously described. However, this issue, associated with host immunotolerance, had been bypassed by immunizing prion protein knock-out mice (PrP0/0) (Bueler et al., 1992) with pathological prion proteins from scrapie (PrPsc) (Betemps and Baron, 2001; Prusiner et al., 1993). We therefore decided to generate anti-α-synuclein antibodies by immunizing a subpopulation of C57BL/6S mice presenting a natural deletion of the α-synuclein locus with the recombinant human protein. In contrast to wild-type C57BL/6J and BALB/c mice, immunization of the α-synuclein-null C57BL/6S line resulted in very high amounts of circulating antibodies, mainly consisting of IgG. Even the BALB/c mice, commonly used in the production of monoclonal antibodies, presented amounts of circulating IgG two or three times lower than the ones obtained with the C57BL/6S line. These results suggest that the natural deletion of snca in the C57BL/6S host allowed the recognition of the immunogen as a non-self protein thus generating a strong polyclonal immune response.

Following the first immunization protocol, an indirect ELISA assay using an anti-IgG (H+L) secondary antibody was used to quantify amounts of circulating pAb, and to screen hybridomas. These assays led to the selection of a single IgG (antibody AS11) among a very large proportion of anti-α-synuclein IgM antibodies. Indeed, since light chains are shared by all immunoglobulin classes, an anti-IgG (H+L) secondary antibody may also recognize other immunoglobulins including IgM. In an attempt to avoid such a cross-reaction, a double screening was performed for the second immunization protocol, using a γ heavy chain specific anti-mouse IgG antibody and a μ heavy chain specific anti-mouse IgM antibody. Thus, this double-titration of anti-α-synuclein pAb highlighted that the rates of circulating IgG were five times greater than those of...
IgM in sera of C57BL/6S. Nevertheless, after the splenocytes fusion of the C57BL/6S mouse presenting the highest ratio of circulating IgG/IgM, we managed to produce only IgM secreting clones, failing to generate any secreting IgG. The duration (58 days for the first protocol and 75 days for the second) and the number of boosts (4) done during our immunization protocols should have allowed class switching from IgM to IgG. Although the reasons for the large proportion of IgM still remain unclear, they seem to be associated with the fusion or cloning steps, rather than the immunization protocols. Similar difficulties were previously mentioned (Williamson et al., 1996) during studies to generate monoclonal antibodies against the prion protein. In that study, serum IgG titer against human PrP were detected after immunization of PrP0/0 knock-out mice with PrPSc, but they failed to establish stable hybridoma cell lines secreting monoclonal antibodies against PrP. The level of PrP expression in the myeloma cell line used for fusion might possibly be a limiting element for the survival of hybrid clones producing monoclonal anti-PrP antibodies.

In our immunization strategies, the conformation of the recombinant human α-synuclein did not seem to have an influence on its immunogenicity. When compared to untreated α-synuclein, the treatments of immunogen in high-salt or acidic conditions to promote the formation of α-synuclein fibrils did not increase the amount of α-synuclein antibodies in the sera of immunized mice. Qualitatively, it was much more difficult to evaluate the exact impact of immunogen conformation on the epitope recognized by antibodies generated. However, we noticed that immunizations of C57BL/6S with untreated recombinant α-synuclein versus an aggregated antigen did not lead to the selection of antibodies with specificity for one or the other of these two forms.

We next focused on the characterization of AS11, the anti-α-synuclein IgG generated by immunizing C57BL/6S mice with recombinant human α-synuclein fibrillized under acidic conditions. These studies aimed to provide functional information for the use of mAb AS11 as a tool to detect α-synuclein in Western-blot, immunofluorescence and immunohistochemical assays.
By Western-blot analysis, the antibody AS11 could detect both recombinant wild-type α-synuclein and cerebral A53T α-synuclein extracted from young, asymptomatic transgenic mice over-expressing the A53T mutated human transgene (Giovanelli et al., 2002). In contrast, no band corresponding to α-synuclein was detected by AS11 in cerebral extracts from the C57BL/6S ΔsncA line, proving the specificity of this antibody. The specificity of AS11 was also supported by immunofluorescence assays performed on neuroblastoma cell lines and NSCs. In fact, the expression of human α-synuclein in SH-SY5Y and NSCs derived from mice over-expressing A53T α-synuclein was associated with a diffuse cytoplasmic immunostaining of α-synuclein by AS11. No staining was observed in NSCs derived from C57BL/6S ΔsncA mice. Moreover, these Western-blot and immunofluorescence assays also demonstrated the ability of antibody AS11 to detect both cerebral α-synuclein under denaturing conditions and cytoplasmic α-synuclein in neuronal cell cultures.

The characterization of murine NSCs over-expressing human A53T α-synuclein allowed us to describe expression of the transgene under the control of the PrP promoter. Monoclonal antibody AS11 immunostained human A53T mutated α-synuclein in undifferentiated cultures, also labeled by nestin, a marker of precursors cells of the nervous system. This expression of the α-synuclein transgene in undifferentiated NSCs taken from 13.5-day-old embryos of line M83 was expected since transcripts of the murine PrP gene are detectable in the developing brain and spinal cord by 13.5–24 days (Manson et al., 1992). Moreover, low but detectable amounts of cellular PrP (PrPc) were previously detected in neonatal C57BL/6J mice, presenting a deletion of the murine PrP gene (in asymptomatic brains of mouse transgenic line M83) were recognized as a defining morphological feature of MSA (Dickson et al., 2009). In our NSCs cultures after 6 days of differentiation, we observed that α-synuclein was predominantly present in β-III-tubulin-positive neurons. Alongside this population of neurons, some cells expressing the astrocytic marker GFAP could also be detected by immunofluorescence.

As demonstrated by our immunohistochemical studies, antibody AS11 could also be used as a diagnostic tool. To date, the definitive diagnosis of synucleinopathies remains based on post-mortem analysis, underlying the need to have specific and sensitive antibodies able to detect α-synuclein in embedded brain sections. The glial cytoplasmic inclusions found in the pons, composed of filamentous, insoluble α-synuclein stained by antibody AS11, are recognized as a defining morphological feature of MSA (Dickson et al., 1999). Antibody AS11 can also be used for the definitive diagnosis of PD by labelling Lewy bodies and Lewy neurites, which are intracellular proteinaceous inclusions mostly composed of polymers of fibrillized α-synuclein (Beta et al., 1998). Finally, using cellular and mouse models, we have demonstrated that antibody AS11 was able to detect several forms of α-synuclein. Both soluble human wild-type (in SH-SY5Y) and A53T α-synuclein (in asymptomatic brains of mouse transgenic line M83) were recognized by AS11. In addition, it was shown that AS11 could also bind pathological filamentous forms of the protein in human disease brains or in SH-SY5Y cells over-expressing recombinant WT human α-synuclein following treatment with rotenone. A previous study established the selection of three mAbs (Syn 505, Syn 506 and Syn 514) that had the ability to detect aggregated α-synuclein in pathological inclusions (Duda et al., 2002; Giovanelli et al., 2000a; Waxman et al., 2008). These antibodies preferentially recognized N-terminal epitopes in complex conformations (Waxman et al., 2008) that could possibly be connected to the pathological changes of α-synuclein occurring during its fibrillization. Western-blot analysis with synthetic peptides demonstrated that antibody AS11 also recognized an epitope in the N-terminal region of α-synuclein (aa 1–60). Moreover, like antibody AS11 which was raised against fibrillized α-synuclein, the antibodies Syn 505, Syn 506 and Syn 514 were all generated against oxidized/nitrated forms of recombinant α-synuclein that mimicked pathological species of the protein (Duda et al., 2002; Giovanelli et al., 2000a; Waxman et al., 2008). Nevertheless, as previously mentioned, it remains difficult to understand the manner in which modifications of the immunogen conformation could influence the recognition antibodies patterns. Although it is unknown in our experiments what is the respective influence of the immunization of α-synuclein deleted mice or/and the fibrillized presentation of recombinant α-synuclein used as immunogen, it is noteworthy that the AS11 antibody recognizes an N-terminal epitope. Whereas most human-specific antibodies recognize the more divergent C-terminal part of the protein, this emphasizes that our strategy could usefully allow to expand the panel of available monoclonal antibodies.

In conclusion, a strong immune response against α-synuclein could be raised by immunizing C57BL/6S mice deleted for snca. Immunoglobulin G AS11 was selected for its significant specificity and reactivity in techniques commonly used to study and describe pathological alterations of α-synuclein in neurodegenerative disease such as PD, MSA or DLB. Antibody AS11 could serve as a sensitive probe for detection of α-synuclein in Western-blot, immunohistochemistry and immunofluorescence assays.

Acknowledgments

We gratefully thank Alain Dorier for his expertise in antibody production and Emilie Antier for performing immunization and animal care. We also want to thank Véronique Métayer, Caroline Seiner and Marjorie Ducreux for their great technical assistance and Bénédicte Berger for helpful discussions.

A.-L.J.M. was supported by a training grant (CIFRE 2006/1050) from the ANRT (Association Nationale de la Recherche et de la Technologie).

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Please cite this article in press as: Mougenot A-LJ, et al. Production of a monoclonal antibody, against human α-synuclein, in a subpopulation of C57BL/6J mice, presenting a deletion of the α-synuclein locus. J Neurosci Methods (2010), doi: 10.1016/j.jneumeth.2010.08.010
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