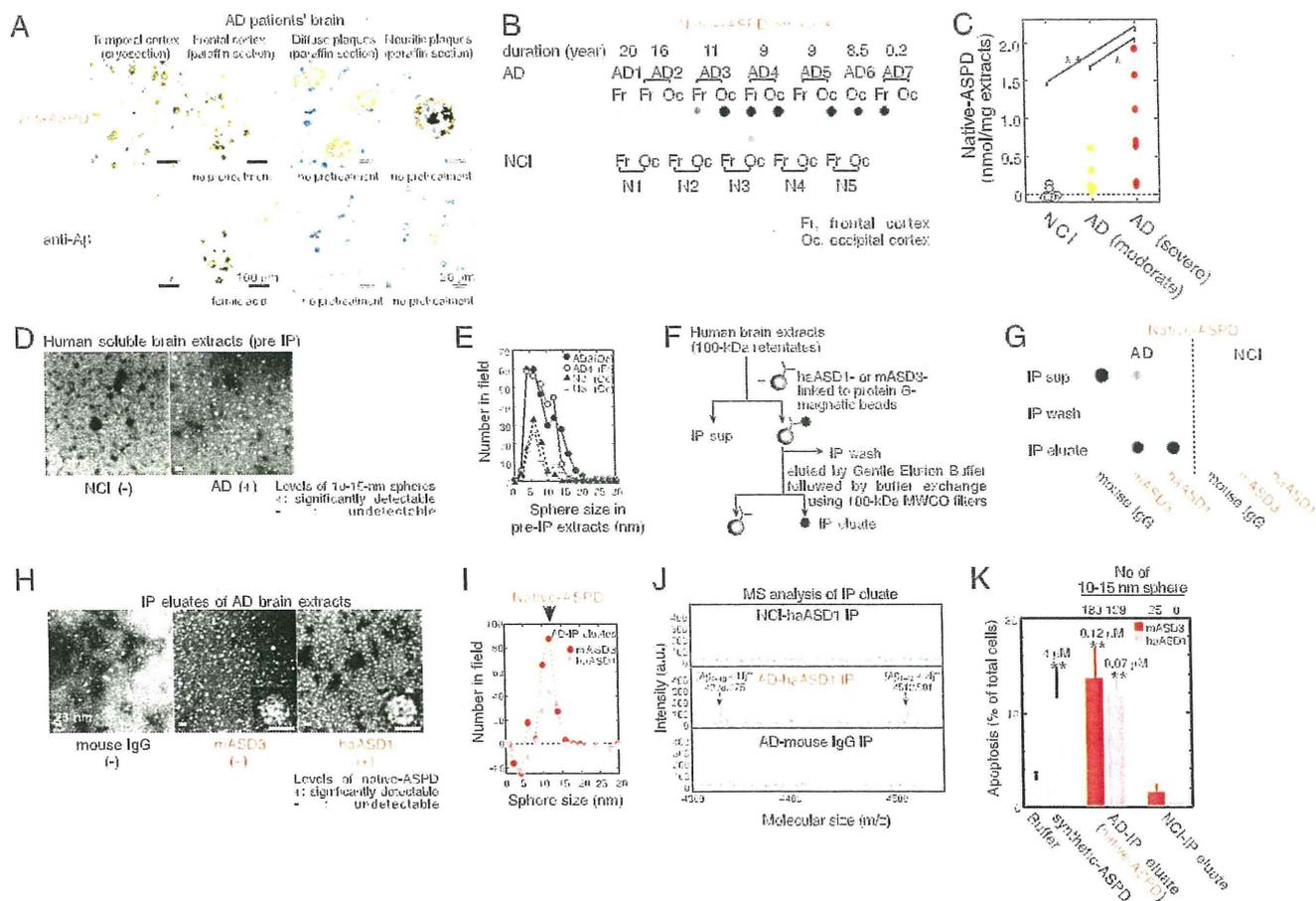


## Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients



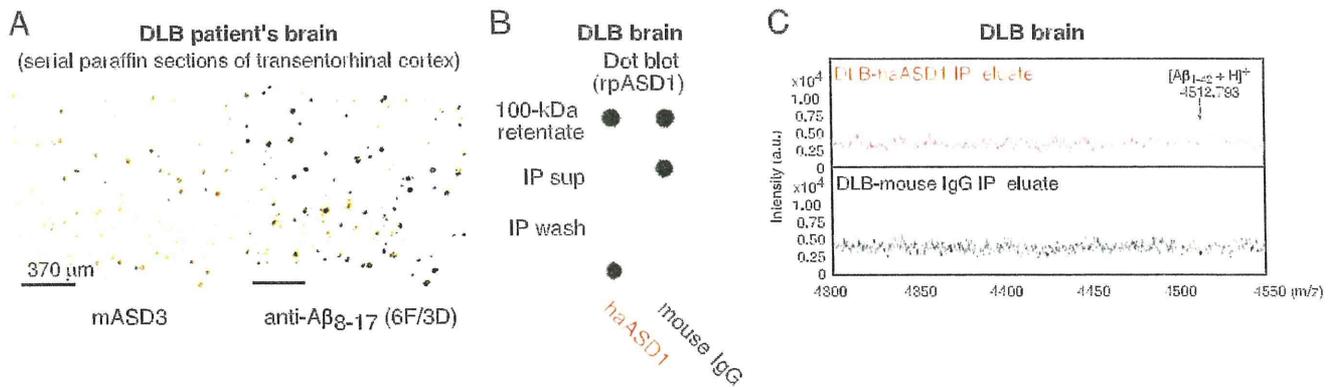
**FIGURE 2. Isolation of native ASPDs.** *A*, AD brains were stained with rpASD1 (5  $\mu$ g/ml) or anti-A $\beta$ 1–42 C-terminal antibody (0.5  $\mu$ g/ml; 2  $\mu$ g/ml for cryosections). *B* and *C*, dot blotting of 100-kDa retentates ( $>$ 100 kDa) of AD or NCI brain extracts (1  $\mu$ g of soluble extracts/dot) using rpASD1 (Scheffé post hoc test; \*\*,  $p < 0.0011$ ; \*,  $p = 0.0388$ ). Fr, frontal cortex; Oc, occipital cortex. *D* and *E*, TEM images (*D*) and particle analysis of 100-kDa retentates ( $n = 3$ ; 10 randomly selected fields) (*E*). *F* and *G*, method for immunoprecipitation (IP) (*F*) and dot blotting (using rpASD1) of IP supernatants (sup), wash, and eluate fractions. IPs were performed using haASD1, mASD3, or mouse IgG (*G*). *H* and *I*, TEM images (inset, bar, 10 nm) (*H*) and particle analysis of IP eluates ( $n = 3$ ; 15 randomly selected fields, background (a small amount of spheres  $<$  10 nm contained in eluate with buffers)-subtracted data are shown) (*I*). *J*, representative MALDI-TOF/MS data. A $\beta$ -(1–40) and A $\beta$ -(1–42) were detected only in native ASPDs at theoretical monoisotopic mass values ([A $\beta$ -(1–40) + H]<sup>+</sup>, 4328 Da; [A $\beta$ -(1–42) + H]<sup>+</sup>, 4512 Da) as observed in synthetic A $\beta$  peptides. *K*, toxicity of isolated native ASPDs toward primary rat septal neurons (mean  $\pm$  S.D.; Scheffé post hoc test, \*\*,  $p < 0.0001$ , compared with buffer,  $n > 8$ ) correlated with the 10–15-nm sphere number determined as in *I*. Neurons treated with NCI-IP eluates showed only background levels of apoptosis similar to those of neurons treated with buffers. Inset, synthetic or native ASPD amounts in A $\beta$  monomer concentrations.

with insoluble fractions of AD brains extracted with SDS or formic acid (supplemental Fig. S3A). Furthermore, this insoluble fraction produced broad smears in Western blots of A $\beta$ , as is usually observed with fibrils (42) (supplemental Fig. S3B). In contrast, the ASD antibodies reacted only with soluble fractions of AD brains (supplemental Fig. S3A) in which the human ASPD counterpart was actually present, as described below (see under “Isolation of Native ASPD from Brains of AD Patients”). These results collectively indicate that the ASD antibodies detect a human ASPD counterpart, namely native ASPD, associated with plaques and neurites in AD brains. In subsequent work, we used monoclonal mASD3 and haASD1 for isolating ASPDs, because of their high affinity, and polyclonal rpASD1 for detecting ASPDs (except Fig. 3A; see also “Immunoprecipitations” under “Experimental Procedures”).

**Isolation of Native ASPD from Brains of AD Patients**—The tissue fractionation study revealed that native ASPDs are recovered in soluble fractions of AD brains. To investigate the amount of native ASPD, we prepared soluble fractions of AD

brains ( $n = 7$ ; age  $85.6 \pm 3.1$  years, brain weight  $1025 \pm 104$  g) and NCI ( $n = 5$ ; age  $72.6 \pm 9.5$  years, brain weight  $1236 \pm 64$  g) by means of a nondenaturing procedure using solutions of physiologic ionic strength and pH without detergents. We then obtained 100-kDa retentates of the soluble fractions to concentrate native ASPDs (larger than 100 kDa) and to eliminate other A $\beta$  assemblies smaller than 100 kDa (as performed in Fig. 1A). The 100-kDa retentates of AD brains thus obtained had high levels of rpASD1-reactive substances, but those of NCI brains had very low or negligible reactivity (Fig. 2B). Consistent with the above data, much higher numbers of spheres sized 10–15 nm were present in 100-kDa retentates of AD patients than in those of NCI (Fig. 2, D and E). These results suggest that rpASD1-reactive 10–15-nm spheres in 100-kDa retentates of AD are native ASPD candidates. We then immunisolated native ASPDs (Fig. 2F) from large amounts of AD-derived 100-kDa retentates using two monoclonal antibodies, haASD1 and mASD3 (Fig. 2, G–J). These antibodies were chosen for their extremely high affinity for ASPD ( $K_d < 10^{-12}$  M) and for their

### Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients



**FIGURE 3. Native ASPDs exist in DLB brains.** *A*, immunostaining using mASD3 (2.5  $\mu$ g/ml) and anti-A $\beta$ 8–17 (pretreated with formic acid; 1:100; DAKO). *B*, IP was performed with haASD1 or mouse IgG as in Fig. 2*F* using 100-kDa retentates (4  $\mu$ g of soluble brain extracts/IP). Dot blotting (0.04  $\mu$ g/ml rpASD1) of 100-kDa retentates (2  $\mu$ g of soluble brain extracts/dot), IP supernatants (*sup*), wash, and eluate is shown. *C*, representative MALDI-TOF/MS data.

recognition of different epitopes (Table 1). Judging from the results of quantification of dot blots using rpASD1 (Fig. 2*G*), we obtained about 43 pmol of native ASPDs (expressed as A $\beta$  monomer concentration) from 1 g of AD brain tissues ( $n = 6$ ). The rpASD1 reactivity in the IP eluates was considered to be mostly due to the 10–15-nm spheres, because the number of spheres counted by TEM (Fig. 2*H*) ( $1.0 \times 10^{10}$  10–15-nm sphere/ $\mu$ l estimated from the number of spheres in Fig. 2*K*,  $n = 6$ ) was very similar to the amount of rpASD1-reactive ASPD obtained from dot blots ( $1.1 \times 10^{10}$  native ASPD/ $\mu$ l based on the ASPD concentration in Fig. 2*K*,  $n = 8$ ). This means that rpASD1-reactive 10–15-nm spheres were selectively isolated by a combination of 100-kDa retention and IP. Indeed, as shown by the TEM data (Fig. 2*H*), the non-ASPD small-sized spheres (<10 nm) that had been present in large amounts in 100-kDa retentates of AD and NCI were largely eliminated by the IP procedure (compare Fig. 2, *I* with *E*). Accordingly, we successfully isolated native ASPDs, consisting of 10–15-nm spheres (>95%; Fig. 2, *H* and *I*), from 100-kDa retentates of AD. In contrast, native ASPD-like assemblies were scarcely detected in IP eluates from 100-kDa retentates of NCI (Fig. 2, *G* and *K*). We next examined whether native ASPDs consisted of A $\beta$ . Mass spectrometric analysis showed that singly charged ions corresponding to A $\beta$ (1–42) and A $\beta$ (1–40) were detected in native ASPDs (Fig. 2*J*). These results collectively demonstrate that 10–15-nm spherical A $\beta$  assemblies isolated from AD brains are native ASPDs. Notably, anti-pan A $\beta$  6E10 could not immunoprecipitate native ASPDs (data not shown), probably because of its weak affinity for ASPDs ( $K_d \approx 10^{-9}$  M) compared with ASD antibodies ( $K_d < 10^{-12}$  M) (Table 1). We also confirmed that anti-pan oligomer A11 antibody failed to detect native ASPDs (supplemental Fig. S4).

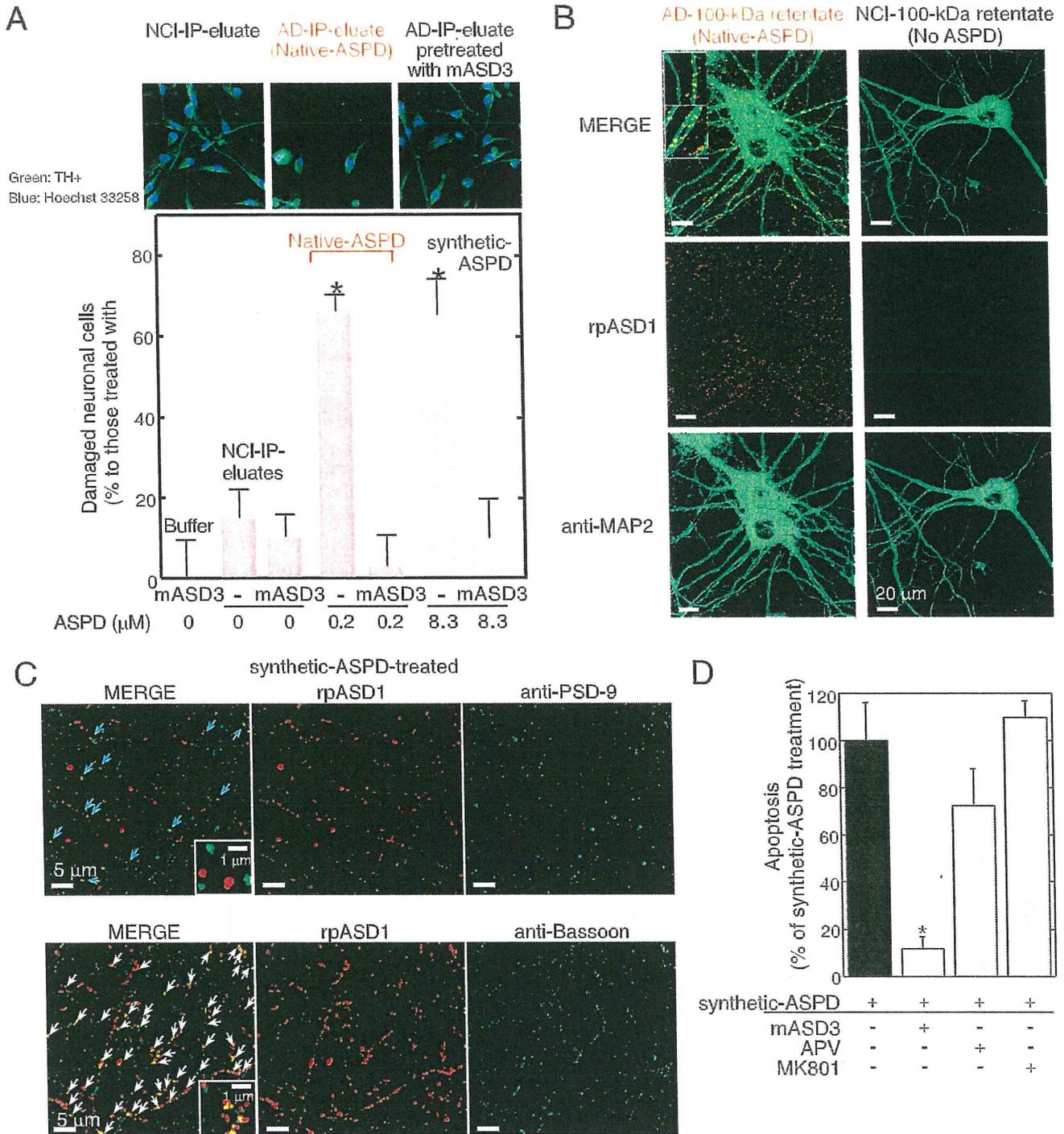
Having isolated native ASPDs selectively from human AD brains, we next examined whether they elicited neurodegeneration of rat primary neuronal cells. Surprisingly, AD-derived native ASPDs were even more toxic than synthetic ASPDs (Fig. 2*K*). These results collectively demonstrate that we have newly isolated A11-negative, high mass assemblies that cause neuronal cell death and that differ in mass and surface tertiary structure from other reported nonfibrillar A $\beta$  assemblies.

**Native ASPD Amount Correlates with the Pathologic Severity of AD**—We next examined whether the amount of native ASPD correlated with the pathologic severity of AD brains. Larger amounts of native ASPD were present in AD patients with severe pathology (diagnosed “C” according to the CERAD criteria (43)) than in AD patients with moderate pathology (diagnosed “B”) (Fig. 2*C*). Furthermore, in AD patients with severe pathology, significantly higher amounts of native ASPD were detected in the frontal or temporal cortices ( $7.2 \pm 1.5$  nmol/g brain tissue,  $n = 3$ , Scheffé post hoc test  $p = 0.0012$ ) than in the cerebellum ( $0.14 \pm 0.1$  nmol/g brain tissue). The result is consistent with previous findings that the cerebellum in AD is pathologically less affected (44, 45).

The above observations suggest the involvement of native ASPDs in neurodegeneration of AD brains. We therefore examined brains of patients suffering from DLB, the second most frequent cause of cognitive decline associated with neurodegeneration in the elderly (46, 47), because the majority of DLB brains have been shown to have AD-type pathology, including plaques (46–48). Interestingly, native ASPDs were also isolated from DLB brains (Fig. 3, *A–C*).

**AD-derived Native ASPDs Cause Severe Degeneration of Human Neuronal Cells**—To further elucidate the relationship between neuronal loss and native ASPDs, we first examined whether native ASPDs induce degeneration of human mature neuronal cells. Because studies using human primary neurons are problematic for ethical and practical reasons, cells with neuronal properties were induced from human bone marrow stromal cells (MSCs) (49). Initially, postmitotic neuronal cells were induced from human MSCs (>95% were neuron-specific MAP2ab-positive cells without glia) (49). Treatment of these cells with glial cell line-derived neurotrophic factor promoted their maturation into functional neuronal cells (49). We found that a 2-day treatment of the human MSC-derived functional neuronal cells with isolated native ASPDs caused severe degeneration, whereas IP eluates from NCI brains had no effect (Fig. 4*A*). In addition, pretreatment with mASD3 antibody (100  $\mu$ g/ml) significantly blocked this toxicity (Fig. 4*A*), as observed in the case of the 158–669-kDa ASPDs (supplemental Fig. S5.4), dem-

### Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients



**FIGURE 4. Characterization of native and synthetic ASPD-induced toxicity.** A, IP was performed using haASD1 as in Fig. 2F. Human neuronal cells were treated for 2 days with AD or NCHIP eluates, with or without 2-h mASD3 (100  $\mu$ g/ml) pretreatment. Nondamaged cells were counted after tyrosine hydroxylase (TH<sup>+</sup>) and Hoechst 33258 staining. The ratio of damaged cells to neuronal cells treated with buffer alone (mean  $\pm$  S.D.) is shown (Scheffé post hoc test; \*,  $p < 0.0001$ ,  $n = 5$ ). Neuronal cells treated with mASD3 alone or NCHIP eluates showed only background levels of damaged cells similar to those in the case of cells treated with buffer. B and C, mature rat hippocampal neurons (24 DIV in B and 19 DIV in C) were incubated for 30 min either with 100-kDa retentate of AD (containing 0.8  $\mu$ M native ASPDs) or NCI (no native ASPD detected) brain extracts in B or with 0.5  $\mu$ M 158–669-kDa ASPDs (prepared from A $\beta$ (1–42); see Fig. 1A) in C. Bound ASPDs were detected by rpASD1, as in Fig. 1D. Punctate labeling was found primarily on neurites and surrounding cell bodies of neurons treated with native or synthetic ASPDs, but it was hardly detectable in neurons treated with the NCI retentates. A representative high power view is shown in the inset (B; bar, 5  $\mu$ m). Neurons were co-stained with an antibody against anti-MAP2 in B, against a postsynaptic marker PSD-95 in C (upper panels), or against a presynaptic marker bassoon in C (lower panels). Z stack images are shown (except lower panels in C) as in Fig. 1D. Bound ASPDs did not co-localize with PSD-95 but were concentrated with bassoon (white arrows in C), although they were occasionally localized in close proximity to PSD-95 (blue arrows in C). D, mature rat hippocampal neurons (21 DIV) were treated with 1  $\mu$ M 158–669-kDa ASPDs for 2 days, with or without pretreatment (100  $\mu$ g/ml mASD3 for 2 h; competitive (APV) or uncompetitive (MK801) NMDA-R antagonists (10  $\mu$ M) for 30 min). Data represent mean  $\pm$  S.D. (Scheffé post hoc test; \*,  $p = 0.0039$ , compared with synthetic ASPDs; synthetic ASPDs,  $n = 7$ ; synthetic ASPDs + APV or MK801,  $n = 5$ ; synthetic ASPDs + mASD3,  $n = 4$ ).

## Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients

onstrating that the observed neuronal cell death was caused by native ASPDs.

We then examined whether native ASPDs bind mature rat hippocampal neurons, as is observed in the case of synthetic ASPDs (Fig. 1D and supplemental Fig. S5B). Binding of AD-derived native ASPDs to 24-DIV mature rat hippocampal neurons was detected with rASD1 most intensely in neurites and also to some extent in cell bodies (Fig. 4B). These results suggest that, despite the difference in dose dependence of neurotoxicity (Figs. 2K and 4A), native and synthetic ASPDs share essentially the same mechanism of neurotoxicity, *i.e.* they have the same surface tertiary structure that is responsible for exerting the toxicity. We speculate that the apparent difference in dose dependence might be attributed to differences in molecular compositions, but testing this idea will require further analyses using large amounts of isolated native ASPDs.

**Mode of Native ASPD Neurotoxicity Is Different from That of Other Reported A $\beta$  Assemblies**—The above results (Fig. 4, A and B) show that native ASPDs cause neuronal cell death, possibly by binding to neuronal cell surfaces. We therefore examined ASPD-binding sites on mature neurons to elucidate the molecular basis of native ASPD neurotoxicity. As shown in the high power images in Fig. 4B (*inset*), bound native ASPDs appeared to protrude from the MAP2 staining of dendrites. Essentially the same results were obtained with the binding of synthetic ASPDs (supplemental Fig. S5B (*inset*)). Because of the limited availability of native ASPDs, we employed synthetic ASPDs for further analysis, as synthetic and native ASPDs share essential properties. Consistent with the above observation, the binding of synthetic ASPDs did not co-localize with a postsynaptic marker, PSD-95 (Fig. 4C, *upper panel*), although it was occasionally detected in close proximity to PSD-95 (*blue arrows in C*). Instead, ASPD-binding sites appeared to be concentrated at presynaptic sites stained by the antibody against a presynaptic marker, bassoon (*white arrows in Fig. 4C, lower panel*).

Although previous studies using cell or slice culture systems have found that A $\beta$  assemblies such as dimers, ADDLs and A $\beta$ Os bind postsynapses and depend on postsynaptic signaling mechanisms for exerting synaptotoxicity (23), the presynaptic binding of ASPDs apparent in Fig. 4B suggests that ASPD neurotoxicity would not require postsynaptic signaling mechanisms such as the *N*-methyl-D-aspartate glutamate receptor (NMDA-R) pathway. Indeed, neither a competitive (APV) nor an uncompetitive (MK801) NMDA-R antagonist inhibited synthetic ASPD-induced neurodegeneration (Fig. 4D). As noted above, native and synthetic ASPDs share the common surface tertiary structure responsible for exerting the toxicity. Therefore, the findings obtained with synthetic ASPDs (Fig. 4, C and D) strongly suggest that native ASPDs cause neuronal cell death through presynaptic target(s) on mature neurons. Furthermore, these observations are consistent with the findings indicating that native ASPDs have a distinct surface tertiary structure from other reported A $\beta$  assemblies and support the hypothesis that native ASPDs have a different target(s) from other A $\beta$  assemblies.

## DISCUSSION

A $\beta$  assemblies are considered to acquire surface tertiary structures that are not present in physiologic A $\beta$  monomers and that induce synaptic impairment and neuronal loss through interactions with neuronal cells. Therefore, as recently suggested (12), it is reasonable to classify seemingly different A $\beta$  assemblies in terms of their immunoreactivity to antibodies that recognize particular surface tertiary structure. Because the surface tertiary structure mediates the binding of A $\beta$  assemblies to their target(s) and is therefore responsible for exerting the toxic effects, A $\beta$  assemblies having distinct surface tertiary structures are likely to have distinct mechanisms of neurotoxicity and may contribute differently to the disease development. Here we have demonstrated the existence of patient-derived native ASPDs by selectively immunisolating them from AD and DLB brains (Figs. 2 and 3) using ASPD tertiary structure-dependent antibodies (Fig. 1 and Table 1). The native ASPDs (>100 kDa) thus obtained are larger in mass than AD-derived A $\beta$  dimers and other reported assemblies such as 12-mers (53~60 kDa; ADDLs, globulomer, A $\beta$ \*56) or A $\beta$ Os (~90 kDa) (supplemental Table S1). More importantly, native ASPDs are considered to have a distinct surface tertiary structure from those other assemblies because they differ in immunospecificity, as illustrated by the fact that ASPD tertiary structure-dependent antibodies showed minimal reactivity with the 100-kDa filtrate containing monomers and dimers (Fig. 1A) or with ADDLs (supplemental Fig. S1A) (16) in dot blots. Additionally, anti-pan oligomer A11 antibody (22) recognized A $\beta$ Os but not synthetic ASPDs (Fig. 1B) or native ASPDs (supplemental Fig. S1). Finally, anti-A $\beta$  N-terminal antibodies such as 82E1 blocked the synaptotoxicity of AD-derived dimers (30) but failed to block synthetic ASPD-induced neurodegeneration (supplemental Fig. S5A). These results all indicate a difference in the surface tertiary structure between these assemblies and ASPDs.

As for the cellular basis of the A $\beta$ -induced synaptic changes, previous studies have suggested the involvement of postsynaptic signaling mechanisms (23). For example, the binding of ADDLs and A $\beta$ Os has been reported to co-localize with PSD-95 (19, 23). As expected from the postsynaptic locale of their binding, ADDLs bind close to or at NMDA-R (23), and NMDA-R antagonists inhibit ADDL-induced dendritic changes (23), reactive oxygen species formation (50), and insulin receptor impairment (51). NMDA-R antagonists have also been reported to inhibit A $\beta$  dimer-induced synaptic loss (24, 30). Interestingly, cellular prion protein, which interacts with NMDA-R (52), has recently been reported to serve as a high affinity postsynaptic receptor mediating ADDL-induced synaptic dysfunction (53). Taken together, these studies are consistent with the idea that A $\beta$  dimers, ADDLs, and A $\beta$ Os perturb postsynaptic transmission (19, 23, 30).

We found that, unlike the above A $\beta$  assemblies, ASPDs bind presynaptic target(s) on neurons to induce neurodegeneration (Fig. 4, A–C). This may be reasonable in view of the distinct ASPD surface tertiary structure. Although the actual targets of native ASPDs remain to be elucidated, native ASPDs seem to affect mature neuron-specific molecules or cellular pathways,

## Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients

as synthetic ASPD-induced neurotoxicity appeared to be confined to neurons, being especially active toward mature neurons, but sparing non-neuronal cells and immature neurons (supplemental Fig. S6, A–C). Together, the findings indicate that native ASPDs are patient-derived, A11-negative, high mass A $\beta$  assemblies with a distinct toxic surface that binds presynaptic target(s) on mature neurons, leading to neuronal loss (supplemental Table S1). Although further studies are required to reveal how native ASPDs exert neurotoxicity in the brains of patients with AD, our findings indicate for the first time that presynaptic signaling mechanisms may play a critical role in A $\beta$ -induced neurodegeneration in AD.

Recent *in vivo* as well as *in vitro* studies support the toxicity of nonfibrillar A $\beta$  assemblies and their possible causative roles in the neuropathology of AD (54–56), which is consistent with the dissociation between fibril load and cognitive decline in patients with AD (32, 57, 58). Thus, A $\beta$  assemblies other than fibrils have been considered to be the preferred therapeutic targets for AD (54). However, the nature of the A $\beta$  species and the oligomer state responsible for the pathogenesis remain controversial because of the heterogeneity of A $\beta$  assemblies in terms of A $\beta$  species and oligomer size. It is also unknown how A $\beta$  monomers assemble into oligomers in living human brains. Nevertheless, previous *in vitro* studies have shown that A $\beta$  monomers develop into a variety of assemblies that might represent distinct structural variants (10–13). These studies suggest that assembly may not be a linear process but may be the result of a series of multiple processes involving intermediates from side paths. Taking all the results together, it seems reasonable to assume that the brains of patients with AD contain distinct types of A $\beta$  assemblies with different surface tertiary structures that may play different roles in AD development. Therefore, identification and characterization of all types of A $\beta$  assemblies actually present in brains from humans with AD will be important for understanding the molecular mechanisms underlying the AD progression from the initial step to the symptomatic phase and for the development of therapies based on this understanding. Fractionation studies using oligomer tertiary structure-dependent antibodies as shown here will help to elucidate the assembly process and to determine the A $\beta$  assembly state causing the pathogenesis. We have isolated native ASPDs that cause degeneration of mature human neuronal cells *in vitro* (Fig. 4A) and have shown that the amount of native ASPD is correlated with the pathologic severity of clinically proven AD cases (Fig. 2C).

These findings suggest that native ASPDs might be a candidate for A $\beta$  assemblies that directly cause neuronal loss in the brains of humans with AD. However, it remains to be elucidated whether or not ASPDs play a particular role in the onset or early stage of disease development. Braak and co-workers (59) have compared the expansion of A $\beta$  pathology in whole brain regions between AD cases and nondemented cases with or without A $\beta$ -related pathology. They found that patients with clinically proven AD exhibit late A $\beta$  stages, although the nondemented cases with AD-related pathology show early A $\beta$  stages. Their findings suggest that AD brains develop pathologic A $\beta$  deposition before clinical symptoms become apparent, and this may start much earlier in nonde-

mented patients with AD-related A $\beta$  pathology. Quantitative studies, with the assistance of clinicians, on the brains of people in different A $\beta$  stages, including nondemented people with AD-related A $\beta$  pathology, will be helpful to elucidate if ASPDs play a role in neuronal loss in AD from the early stage of disease development.

Analyses on brains of APP-transgenic mice with or without neuronal loss would also help to elucidate the relationship between ASPDs and neuronal loss. Although the strain does not show neuronal loss, we examined *Tg2576* mice, the most widely used AD-model mice carrying the human Swedish APP mutant (60), by means of immunohistochemistry and IP. ASPD-like assemblies were only minimally detected in the cerebral cortex of *Tg2576* mice (supplemental Fig. S7, A and B); they were not detected up to 14 months and only a very small amount ( $\sim 0.01$  nmol/mg extracts) was detected at 23 months. As previously reported (18, 61), other A $\beta$  assemblies such as dimers and A $\beta$ \*56 were increased in *Tg2576* mice, and total A $\beta$  reached levels comparable with those in human AD (supplemental Fig. S7C). With respect to mice with neuronal loss, in addition to certain APP transgenic mice (28, 29), there is a growing number of other AD-model mice, which have been produced by combining APP mutations with either presenilin-1 mutations (62, 63), Tau protein mutations (64), or nitric-oxide synthase knock-out (65). It should be noted that the mouse is not a perfect model of human AD, but these mice are considered to more closely resemble what occurs in the human brains. Therefore, further analysis to examine whether ASPD-like assemblies are present in these mice, which do show massive neuronal loss, will contribute to establish the relationship between neuronal loss and ASPDs.

In addition to the above, we are currently seeking to establish a direct link between native ASPDs and neuronal loss in brains from humans with AD by searching for the toxic target(s) of ASPDs on mature neurons. The identification of native ASPDs and availability of the toxicity-neutralizing antibodies should facilitate a mechanistic understanding of the cellular basis of neuronal cell loss in AD, as well as the development of therapies based on this understanding.

*Acknowledgments*—We thank Drs. George R. Martin, Takaomi C. Saido, Sangram S. Sisodia, R. Yu, Y. Fukazawa, D. Masui, M. Hoshino, H. Hara, and A. Sakai, for critical discussions; Dr. Charles G. Glabe for providing control blots for A11 antibody through Invitrogen; and Drs. T. Nirasawa, S. Horie, H. Kinoshita, S. Miyama, Y. Ogawa, and N. Takino for technical assistance.

## REFERENCES

1. Ross, C. A., and Poirier, M. A. (2005) *Nat. Rev. Mol. Cell Biol.* 6, 891–898
2. Selkoe, D. J. (1991) *Neuron* 6, 487–498
3. Lansbury, P. T., and Lashuel, H. A. (2006) *Nature* 443, 774–779
4. Iwatsubo, T. (2007) *Neuropathology* 27, 474–478
5. Soto, C., and Estrada, L. D. (2008) *Arch. Neurol.* 65, 184–189
6. Chiti, F., and Dobson, C. M. (2009) *Nat. Chem. Biol.* 5, 15–22
7. Hardy, J., and Selkoe, D. J. (2002) *Science* 297, 353–356
8. Tanzi, R. E., and Bertram, L. (2005) *Cell* 120, 545–555
9. Saido, T. C., and Iwata, N. (2006) *Neurosci. Res.* 54, 235–253
10. Klein, W. L., Stine, W. B., Jr., and Teplow, D. B. (2004) *Neurobiol. Aging* 25, 569–580

## Isolation of Toxic High Mass A $\beta$ Assembly from AD Patients

11. Walsh, D. M., and Selkoe, D. J. (2007) *J. Neurochem.* **101**, 1172–1184
12. Glabe, C. G. (2003) *J. Biol. Chem.* **278**, 29639–29643
13. Roychoudhuri, R., Yang, M., Hoshi, M. M., and Teplow, D. B. (2009) *J. Biol. Chem.* **284**, 4749–4753
14. Walsh, D. M., Lomakin, A., Benedek, G. B., Condron, M. M., and Teplow, D. B. (1997) *J. Biol. Chem.* **272**, 22361–22372
15. Podlisny, M. B., Ostaszewski, B. L., Squazzo, S. L., Koo, E. H., Rydel, R. E., Teplow, D. B., and Selkoe, D. J. (1995) *J. Biol. Chem.* **270**, 9564–9570
16. Lambert, M. P., Barlow, A. K., Chromy, B. A., Edwards, C., Freed, R., Liosatos, M., Morgan, L. E., Rozovsky, I., Trommer, B., Viola, K. L., Wals, P., Zhang, C., Finch, C. E., Krafft, G. A., and Klein, W. L. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 6448–6453
17. Barghorn, S., Nimmrich, V., Strieflinger, A., Krantz, C., Keller, P., Janson, B., Bühr, M., Schmidt, M., Bitner, R. S., Harlan, J., Barlow, E., Ebert, U., and Hillen, H. (2005) *J. Neurochem.* **95**, 834–847
18. Lesné, S., Koh, M. T., Kotilinek, L., Kaye, R., Glabe, C. G., Yang, A., Gallagher, M., and Ashe, K. H. (2006) *Nature* **410**, 352–357
19. Deshpande, A., Mina, E., Glabe, C., and Busciglio, J. (2006) *J. Neurosci.* **26**, 6011–6013
20. Chimón, S., Shaihat, M. A., Jones, C. R., Calero, D. C., Aizazi, B., and Ishii, Y. (2007) *Nat. Struct. Mol. Biol.* **14**, 1157–1164
21. Lacor, P. N., Bunid, M. C., Chang, L., Fernandez, S. L., Gong, Y., Viola, K. L., Lambert, M. P., Velasco, P. T., Pigio, E. H., Finch, C. E., Krafft, G. A., and Klein, W. L. (2004) *J. Neurosci.* **24**, 10191–10200
22. Kaye, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., and Glabe, C. G. (2003) *Science* **300**, 486–489
23. Lacor, P. N., Bunid, M. C., Furlow, P. W., Clemente, A. S., Velasco, P. T., Wood, M., Viola, K. L., and Klein, W. L. (2007) *J. Neurosci.* **27**, 796–807
24. Shankar, G. M., Bloodgood, B. L., Townsend, M., Walsh, D. M., Selkoe, D. J., and Sabatini, B. L. (2007) *J. Neurosci.* **27**, 2866–2875
25. Cleary, J. P., Walsh, D. M., Hofmeister, J. L., Shankar, G. M., Kuskowski, M. A., Selkoe, D. J., and Ashe, K. H. (2005) *Nat. Neurosci.* **8**, 79–84
26. Ashe, K. H. (2001) *Learn. Mem.* **8**, 301–308
27. Hoek, B. J., Jr., and Lamb, B. T. (2001) *Trends Genet.* **17**, S7–S12
28. Calhoun, M. E., Wiederhold, K. H., Abramowski, D., Phinney, A. L., Probst, A., Sturchler-Pierrat, C., Staufenbiel, M., Sommer, B., and Jucker, M. (1998) *Nature* **395**, 755–756
29. Bondolfi, L., Calhoun, M., Frimini, E., Kuhn, H. G., Wiederhold, K. H., Walker, J., Staufenbiel, M., and Jucker, M. (2002) *J. Neurosci.* **22**, 515–522
30. Shankar, G. M., Li, S., Mhtha, T. H., Garcia-Munoz, A., Shepardson, N. E., Smith, I., Brett, F. M., Farrell, M. A., Rowan, M. J., Lemere, C. A., Regan, C. M., Walsh, D. M., Sabatini, B. L., and Selkoe, D. J. (2008) *Nat. Med.* **14**, 837–842
31. Kuo, Y. M., Emmerling, M. R., Vigo-Pelfrey, C., Kasunic, T. C., Kirkpatrick, J. B., Murdoch, G. H., Ball, M. J., and Roher, A. E. (1996) *J. Biol. Chem.* **271**, 1077–1081
32. Lue, J. F., Kuo, Y. M., Roher, A. E., Brachova, L., Shen, Y., Sue, L., Beach, T., Furch, J. H., Rydel, R. E., and Rogers, J. (1999) *Am. J. Pathol.* **155**, 853–862
33. McLain, C. A., Cherny, R. A., Fraser, F. W., Fuller, S. L., Smith, M. L., Beyreuther, K., Bush, A. I., and Masters, C. L. (1999) *Ann. Neurol.* **46**, 860–866
34. Gómez-Isla, T., Hollister, R., West, H., Mui, S., Growdon, J. H., Petersen, R. C., Parisi, J. E., and Hyman, B. T. (1997) *Ann. Neurol.* **41**, 17–24
35. Morrison, J. H., and Hof, P. R. (1997) *Science* **278**, 412–419
36. Larrieu, S., Letenneur, L., Orgogozo, J. M., Fabrigoule, C., Amieva, H., Le Carret, N., Barberger-Gateau, P., and Dartigues, J. F. (2002) *Neurology* **59**, 1594–1599
37. Bouwman, F. H., Schoonenboom, S. N., van der Flier, W. M., van Elk, E. L., Kok, A., Barkhof, F., Blankenstein, M. A., and Selhens, P. (2007) *Neurobiol. Aging* **28**, 1070–1074
38. Hoshi, M., Sato, M., Matsumoto, S., Noguchi, A., Yasutake, K., Yoshida, N., and Soto, K. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6370–6375
39. Lomakin, A., Chung, D. S., Benedek, G. B., Emschner, D. A., and Teplow, D. B. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 1125–1129
40. Demuro, A., Mina, E., Kaye, R., Milton, S. C., Parker, L., and Glabe, C. G. (2005) *J. Biol. Chem.* **280**, 17294–17300
41. Kuwano, R., Miyashita, A., Arai, H., Asada, T., Imagawa, M., Shoji, M., Higuchi, S., Urolami, K., Kabita, A., Takahashi, H., Tsukie, T., Toyabe, S., Akazawa, K., Kanazawa, L., and Ihara, Y. (2006) *Hum. Mol. Genet.* **15**, 2170–2182
42. Kaye, R., Head, E., Sarsosa, F., Saing, T., Cotman, C. W., Neerula, M., Margold, L., Wu, L., Breydo, L., Thompson, L. L., Rasool, S., Gurle, T., Butler, P., and Glabe, C. G. (2007) *Mol. Neurodegener.* **2**, 18
43. Mirra, S. S., Heyman, A., McKeel, D., Sumi, S. M., CRAIN, B. L., Brownlee, L. M., Vogel, F. S., Hughes, J. P., van Belle, G., and Berg, L. (1991) *Neurology* **41**, 479–486
44. Li, Y. T., Woodruff-Pak, D. S., and Trojanowski, J. Q. (1994) *Neurobiol. Aging* **15**, 1–9
45. Kepe, V., Huang, S. C., Small, G. W., Satyamurthy, N., and Barrio, J. R. (2006) *Methods Enzymol.* **412**, 144–160
46. Ince, P. G., and McEath, I. G. (2003) in *Neurodegeneration: The Molecular Pathology of Dementia and Movement Disorders* (Dickson, D., ed) pp. 188–197. ISN Neuro-path Press, Basel
47. Giasson, B. I., Lee, V. M.-Y., and Trojanowski, J. Q. (2004) in *The Neuropathology of Dementia* (Esiri, M., Lee, V. M.-Y., and Trojanowski, J. Q., eds) 2nd Ed., pp. 353–375. Cambridge University Press, Cambridge
48. Kosaka, K. (1990) *J. Neurol.* **237**, 197–204
49. Dezawa, M., Kanno, H., Hoshino, M., Cho, H., Matsumoto, N., Itokazu, Y., Tajima, N., Yamada, H., Sawada, H., Ishikawa, H., Mimura, T., Kitada, M., Suzuki, Y., and Ide, C. (2004) *J. Clin. Invest.* **113**, 1701–1710
50. De Felice, F. G., Velasco, P. T., Lambert, M. P., Viola, K., Fernandez, S. L., Ferreira, S. T., and Klein, W. L. (2007) *J. Biol. Chem.* **282**, 11590–11601
51. Zhao, W. Q., De Felice, F. G., Fernandez, S., Chen, H., Lambert, M. P., Qian, M. L., Krafft, G. A., and Klein, W. L. (2008) *FASEB J.* **22**, 246–260
52. Khosravi, H., Zhang, Y., Tsutsui, S., Hamed, S., Altier, C., Hamid, I., Chen, L., Villemaire, M., Ali, Z., Jirik, F. R., and Zamponi, G. W. (2008) *J. Cell Biol.* **181**, 551–565
53. Laurén, J., Gimbel, D. A., Nygaard, H. B., Gilbert, J. W., and Strittmatter, S. M. (2009) *Nature* **457**, 1128–1132
54. Klein, W. L., Krafft, G. A., and Finch, C. E. (2001) *Trends Neurosci.* **24**, 219–224
55. Bucciantini, M., Giannini, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) *Nature* **416**, 507–511
56. Walsh, D. M., Klyubin, J., Fadeeva, J. V., Cullen, W. K., Anwyl, R., Wolfe, M. S., Rowan, M. J., and Selkoe, D. J. (2002) *Nature* **416**, 535–539
57. Dickson, D. W., and Yen, S. H. (1989) *Neurobiol. Aging* **10**, 402–414
58. Terry, R. D., Masliah, E., Salmon, D. P., Butters, N., DeTeresa, R., Hill, R., Hansen, L. A., and Katzman, R. (1991) *Ann. Neurol.* **30**, 572–580
59. Thal, D. R., Rüb, U., Orantes, M., and Braak, H. (2002) *Neurology* **58**, 1791–1800
60. Hsiao, K., Chapman, P., Hillsen, S., Fekman, C., Harigaya, Y., Yonkin, S., Yang, E., and Cole, G. (1996) *Science* **274**, 99–102
61. Kawarabayashi, T., Younkin, L. H., Saido, T. C., Shoji, M., Ashe, K. H., and Younkin, S. G. (2001) *J. Neurosci.* **21**, 372–381
62. Casus, C., Sergeant, N., Ilier, J. M., Blanchard, V., Wirths, O., van der Kolk, N., Vingdtoux, V., van de Steeg, F., Ret, G., Canton, T., Drobecq, H., Clark, A., Bonici, B., Delacourte, A., Benavides, J., Schmitz, C., Tremp, G., Bayer, T. A., Benoit, P., and Fradier, J. (2001) *Am. J. Pathol.* **165**, 1239–1200
63. Oadley, H., Cole, S. L., Logan, S., Matus, E., Shao, P., Craft, L., Guillezet-Bongacarts, A., Ohno, M., Distelhoff, L., Van Eldik, L., Barry, R., and Vassar, P. (2006) *J. Neurosci.* **26**, 10129–10140
64. Pérez, M., Eibe, E., Rubio, A., Hín, F., Madoñ, M. A., Ramos, P. G., Ferrer, I., Ido, M. T., and Avila, J. (2005) *Neuroscience* **130**, 339–347
65. Wilcock, D. M., Ghahdlou-Journebe, N., Van Nostrand, W. E., Davis, L., Vittek, M. E., and Johnson, C. A. (2009) *J. Neurosci.* **29**, 7957–7965

# Nurr1 Is Required for Maintenance of Maturing and Adult Midbrain Dopamine Neurons

Banafsheh Kadkhodaei,<sup>1</sup> Takehito Ito,<sup>2</sup> Eliza Joodmardi,<sup>1</sup> Bengt Mattsson,<sup>3</sup> Claude Rouillard,<sup>1</sup> Manolo Carta,<sup>3</sup> Shin-Ichi Muramatsu,<sup>4</sup> Chiho Sumi-Ichinose,<sup>5</sup> Takahide Nomura,<sup>5</sup> Daniel Metzger,<sup>6</sup> Pierre Chambon,<sup>6</sup> Eva Lindqvist,<sup>7</sup> Nils-Göran Larsson,<sup>8,10</sup> Lars Olson,<sup>7</sup> Anders Björklund,<sup>3</sup> Hiroshi Ichinose,<sup>2</sup> and Thomas Perlmann<sup>1,9</sup>

<sup>1</sup>Ludwig Institute for Cancer Research, Stockholm Branch, SE-171 77 Stockholm, Sweden, <sup>2</sup>Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan, <sup>3</sup>Wallenberg Neuroscience Center, Lund University, SE-221 84 Lund, Sweden, <sup>4</sup>Department of Neurology, Jichi Medical University, Tochigi 329-0498, Japan, <sup>5</sup>Department of Pharmacology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan, <sup>6</sup>Department of Functional Genomics Institut de Génétique et Biologie Moléculaire et Cellulaire, 67404 Illkirch, France, Departments of <sup>7</sup>Neuroscience, <sup>8</sup>Laboratory Medicine, and <sup>9</sup>Cell and Molecular Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden, and <sup>10</sup>Max Planck Institute for Biology of Ageing, D-50931 Cologne, Germany

Transcription factors involved in the specification and differentiation of neurons often continue to be expressed in the adult brain, but remarkably little is known about their late functions. Nurr1, one such transcription factor, is essential for early differentiation of midbrain dopamine (mDA) neurons but continues to be expressed into adulthood. In Parkinson's disease, Nurr1 expression is diminished and mutations in the *Nurr1* gene have been identified in rare cases of disease; however, the significance of these observations remains unclear. Here, a mouse strain for conditional targeting of the *Nurr1* gene was generated, and *Nurr1* was ablated either at late stages of mDA neuron development by crossing with mice carrying Cre under control of the dopamine transporter locus or in the adult brain by transduction of adeno-associated virus Cre-encoding vectors. *Nurr1* deficiency in maturing mDA neurons resulted in rapid loss of striatal DA, loss of mDA neuron markers, and neuron degeneration. In contrast, a more slowly progressing loss of striatal DA and mDA neuron markers was observed after ablation in the adult brain. As in Parkinson's disease, neurons of the substantia nigra compacta were more vulnerable than cells in the ventral tegmental area when *Nurr1* was ablated at late embryogenesis. The results show that developmental pathways play key roles for the maintenance of terminally differentiated neurons and suggest that disrupted function of Nurr1 and other developmental transcription factors may contribute to neurodegenerative disease.

## Introduction

Adaptation to a changing environment requires plasticity in the adult CNS. However, to ensure that neurons are properly maintained, such plasticity must be balanced against mechanisms that counteract phenotypic instability. Studies of how neurons develop may help to unravel functions important for the stability of nerve cells as factors promoting their differentiation may also contribute to their maintenance. Indeed, many transcription fac-

tors identified for their critical roles during neuronal development continue to be expressed in the postnatal nervous system, raising the possibility that they contribute to the integrity of already differentiated neurons (Hendricks et al., 1999; Vult von Steyern et al., 1999; Kang et al., 2007; Alavian et al., 2008). However, the consequences of adult gene ablation of any of these factors have not yet been reported, and very little is known of their functions in differentiated neurons.

From a clinical perspective, it is of particular interest to identify factors that maintain stability of neurons that are affected in neurodegenerative disorders as loss of phenotype would likely cause or contribute to disease. Parkinson's disease (PD) is characterized by progressive pathology of midbrain dopamine (mDA) neurons of substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA), typically involving deposition of  $\alpha$ -synuclein-rich cytoplasmic protein aggregates termed Lewy bodies. During development, early signaling events induce transcription factors that control the specification and differentiation of mDA neurons (Smidt and Burbach, 2007). Several of these factors, including Nurr1, Lmx1a, Lmx1b, Pitx3, FoxA2, and En1/2, continue to be expressed in the postnatal and adult brain (Zetterström et al., 1996; Smidt et al., 1997, 2000; Albéri et al., 2004; Simon et al., 2004; Kittappa et al., 2007). Nurr1, belonging to a family of ligand-independent nuclear receptors (Wang et al.,

Received Aug. 11, 2009; revised Oct. 17, 2009; accepted Oct. 26, 2009.

This work was supported by grants from the Michael J. Fox Foundation (T.P., A.B., L.O.), Vetenskapsrådet via Linne Center DBRM (T.P.), Grants-in-Aid for Human Frontier Science Program (P.C., D.M., H.I.), Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Ministry of Health, Labor, and Welfare of Japan, and the Japan Science and Technology Agency, Core Research for Evolutional Science and Technology (S.-I.M., H.I.), Vetenskapsrådet (L.O.), Swedish Brain Power (L.G.), Swedish Brain Foundation (L.G.), and the Swedish Parkinson Foundation (L.G.). We are grateful to Johan Ericson and members of the Perlmann and Ericson laboratories for valuable discussions. We thank Andree Dienech and Jean-Marc Bernhart for their help in mouse outcrossing, Naoto Ibara for help in construction of the targeting vector and screening of ES clones, Björn Angelus, and Naomi Takino for their help with AAV vectors, Ulla Jalf for help with histology, and Marie-Louise Ålun for advice on mice handling.

Correspondence should be addressed to either of the following: Thomas Perlmann, Department of Cell and Molecular Biology, Karolinska Institutet, SE-171 77 Stockholm, Sweden, E-mail: thomas.perlmann@ki.se; or Hiroshi Ichinose, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226-8501, Japan, E-mail: hichinose@bio.titech.ac.jp.

DOI:10.1523/JNEUROSCI.3910-09.2009

Copyright © 2009 Society for Neuroscience 0270-6474/09/2915923-10\$15.00/0

2003; Perlmann and Wallén-Mackenzie, 2004), becomes expressed in developing mDA neurons that have just exited the cell cycle and is essential for mDA neuron development because mDA neurons of both the SNc and VTA fail to express dopaminergic markers and newborn *Nurr1*-null mice lack mDA neuron cell bodies and their striatal projections (Zetterström et al., 1997; Castillo et al., 1998; Saucedo-Cardenas et al., 1998). How *Nurr1* regulates target genes in mDA neuron development remains essentially unknown but may involve a functional interaction with the homeobox transcription factor *Pitx3* (Jacobs et al., 2009).

Determining the role of *Nurr1* also in the adult brain is of particular importance because previous studies suggested an association of this protein with PD pathology. *Nurr1* expression is diminished in neurons with  $\alpha$ -synuclein inclusions in postmortem PD brain tissue, and *Nurr1* mutations and polymorphisms have been identified in rare cases of PD (Xu et al., 2002; Le et al., 2003; Zheng et al., 2003; Grimes et al., 2006). However, the significance of genetic lesions remain unclear (Wellenbrock et al., 2003; Hering et al., 2004; Tan et al., 2004). These observations emphasize the importance of elucidating the role of *Nurr1* in more mature mDA neurons by analyzing the consequences of conditional *Nurr1* gene ablation in mice.

## Materials and Methods

**Conditional *Nurr1* gene-targeted mice.** Mouse 129SV genomic library constructed in bacterial artificial chromosome (BAC) was screened by PCR. A BAC clone containing the entire *Nurr1* gene was selected, and a BamHI–MunI fragment containing exon 1 to exon 5 was recloned into a pBluescript II vector. A floxed neomycin cassette was inserted into an internal EcoRI site located in intron 3, and a synthetic loxP sequence was inserted at Sall site located in intron 2. Mouse embryonic stem (ES) cells were electroporated with the targeting vector, and the homologously recombined clones were screened by PCR and Southern blot analysis. ES clones with three loxP sites were selected, and a plasmid expressing Cre DNA recombinase was transiently transfected into the cells. ES cells with two loxP sites without a neomycin cassette were selected by PCR and used for production of chimeric mice.

**Animals.** Mice were kept in rooms with controlled 12 h light/dark cycles, temperature, and humidity, with food and water provided *ad libitum*. All animal experiments were performed with permission from the local animal ethics committee. The generation of dopamine transporter (*DAT*)<sup>Cre</sup> mutant mice has been described previously (Ekstrand et al., 2007). Mice were mated during the night, and the females were checked for vaginal plugs in the morning [day of vaginal plug considered as embryonic day 0.5 (E0.5)].

**1-3,4-Dihydroxyphenylalanine treatment.** Methyl 1, 3,4-dihydroxyphenylalanine (L-DOPA) hydrochloride and the peripheral DOPA decarboxylase inhibitor benserazide HCl (Sigma Aldrich) were dissolved in Ringer's solution immediately before use. L-DOPA was intraperitoneally given every second day at the dose of 2.5 mg/kg combined with 0.625 mg/kg benserazide. Chronic treatment with L-DOPA/benserazide was administered for 50 d, starting at postnatal day 15 (P15). During this period, the mice were carefully observed and weight was measured regularly. Reported hyperactivity was observed in *cNurr1*<sup>DA1<sup>Cre</sup></sup> mice when given a single higher dose of L-DOPA (25 mg/kg L-DOPA, 6.25 mg/kg benserazide).

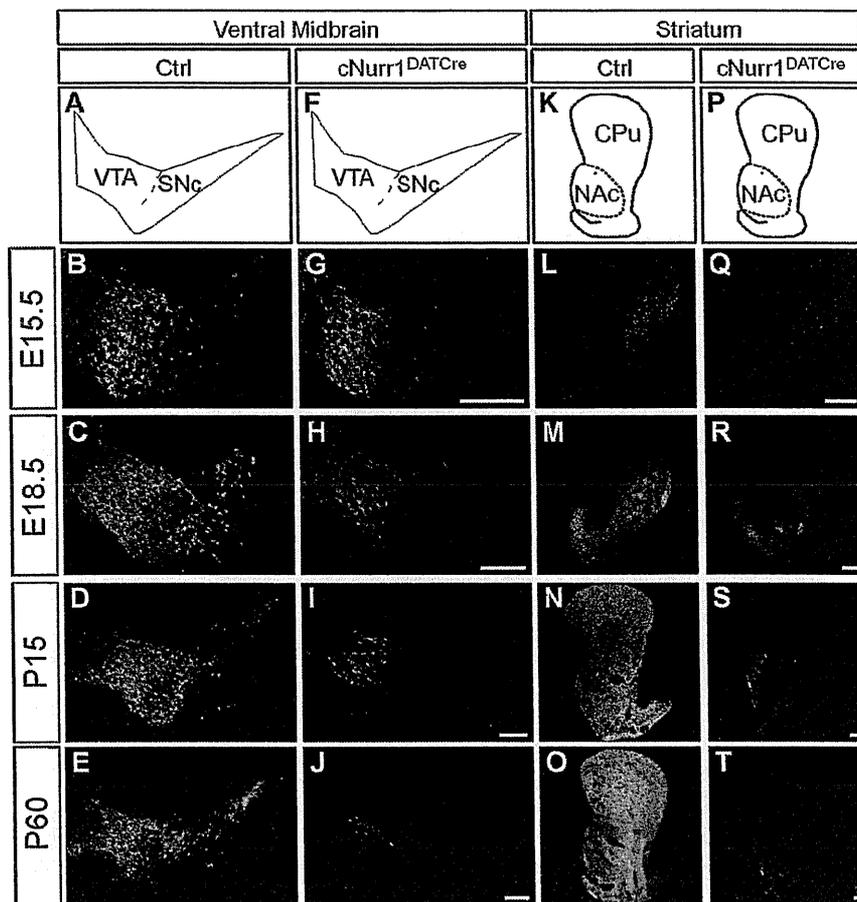
**Histological analyses.** At embryonic stages, embryos were fixed for 2–24 h in 4% phosphate buffered paraformaldehyde (PFA), cryopreserved in 30% sucrose before being embedded in OCT (Sakura Finetek), and cryosectioned at a thickness of 16–20  $\mu$ m onto slides (SuperFrostPlus, Menzel Gläser). For the isolation of brains for immunolabeling at P15 and onward, animals were anesthetized with Avertin (tribromoethanol; 0.5 mg/g) and perfused through the left ventricle with body temperature PBS, followed by ice-cold 4% PFA. The brains were dissected and postfixed overnight in 4% paraformaldehyde and subsequently cryoprotected for 24–48 h in 30% sucrose at 4°C. The brains were serially sectioned on a cryostat or sliding microtome at 10–30  $\mu$ m. Littermates were used in all comparative experiments.

For immunohistochemistry, sections were preincubated for 1 h in blocking solution containing either 10% normal goat sera or 5–10% bovine serum albumin, 0.25% Triton X-100, and 0.01% Na azide in PBS. Primary antibodies diluted in blocking solution were applied overnight at 4°C. After rinses with PBS, biotinylated or fluorophore conjugated secondary antibodies diluted in PBS were applied for 1 h at room temperature. Biotinylated secondary antibodies were followed by incubation with streptavidin-horseradish peroxidase complex (ABC elite kit, Vectastain) for 1 h and subsequent exposure to diaminobenzidine (DAB kit; Vector Laboratories). Primary antibodies and dilution factors were as follows: rabbit anti *Nurr1* (1:100; M196; Santa Cruz Biotechnology), anti-*Nurr1* (1:250; E20; Santa Cruz Biotechnology), rabbit anti tyrosine hydroxylase (TH) (1:500; Pel Freeze), rat anti *DAT* (1:2000; Millipore Bioscience Research Reagents), mouse anti TH (1:200; Millipore Bioscience Research Reagents), rabbit anti vesicular monoamine transporter (VMAT) (1:500; Millipore Bioscience Research Reagents), rabbit anti L-DOPA decarboxylase (AADC) (1:500; Millipore Bioscience Research Reagents), rabbit anti-Cre (1:10,000; Covance Research Products), guinea pig anti *Lmx1b* (1:1000) (Andersson et al., 2006), and rabbit anti *Pitx3* (Smidt et al., 2004). In some cases (anti AADC, anti-VMAT, and anti-*Nurr1*), the blocking steps were performed after antigen retrieval (Dako). Finally, expression was detected by secondary antibodies from Jackson ImmunoResearch. Section images were collected by confocal microscopy (Leica DMIRE2) and bright-field microscopy (Eclipse E1000K; Nikon). Cell counting was performed by counting all SNc DA neurons detected by immunohistochemistry (DAB) in a total of three sections per animal (every 12th tissue section) within the ventral midbrain of animals taken at 4 months after vector injection in both wild type *nurr1*<sup>ΔCre</sup> and *cNurr1*<sup>AAV<sup>Cre</sup></sup> animals. The mean of counted cells per animal was established from both the injected and non-injected sides in each animal, and the relative decrease was calculated as a percent age as described in Results.

**AAV-Cre injections.** Two and a half- to 5 month old animals received one unilateral stereotaxic injection in the right striatum using a 10  $\mu$ l Hamilton microsyringe fitted with a glass pipette tip. The animals were anesthetized with isoflurane, 1  $\mu$ l was injected during 5 min, and the cannula was left in place for an additional 2 min before being slowly retracted. The anteroposterior and mediolateral coordinates from bregma were –2.8 and –1.1 mm, respectively, and the dorsoventral coordinates from the dura were –4.3 mm. Animals were killed 0.5, 1.5, and 4 months after injection, and the brains were isolated.

**Measurement of tissue content for dopamine, serotonin, and their metabolites.** In supplemental Tables 1–3 (available at www.jneurosci.org as supplemental material), tissues were collected from P1, P7, P14, and adult (P48) *w<sup>t</sup>*<sup>DA1<sup>Cre</sup></sup> and *cNurr1*<sup>DA1<sup>Cre</sup></sup> mice. One to 14 d old mice were killed by decapitation, and P48 mice were killed by cervical dislocation. Brains were rapidly removed, chilled in saline (4°C), dissected, frozen on dry ice, and stored at –80°C until use. To process tissues for HPLC and electrochemical detection of monoamines and metabolites, samples were homogenized by sonication in 5 vol or in 30  $\mu$ l of 0.1 M perchloric acid, followed by centrifugation. Endogenous levels of noradrenaline, DA, 3,4-dihydroxyphenylacetic acid, homovanillic acid (HVA), serotonin (5-HT), and 5-hydroxyindoleacetic acid were determined in the supernatants. A reverse column (BAS, C-18, 100.0  $\times$  3.2 mm, 3  $\mu$ m particle diameter) was used for separation. The mobile phase consisted of 0.05 M sodium phosphate/0.03 M citric acid buffer containing 0.1 mM EDTA and was adjusted with various amounts of methanol and sodium 1-octane sulfonic acid. The flow rate was 0.3 ml/min. Monoamines and metabolites were detected using a glassy carbon electrode detector, which as set at +0.7 V versus an Ag/AgCl reference electrode. Resultant peaks were measured and compared with repeated control samples containing fixed mixed amounts of compounds of interest.

In supplemental Table 4 (available at www.jneurosci.org as supplemental material), all animals were killed, and striatum and cortex were rapidly dissected out, frozen on dry ice, and stored at –80°C. To determine monoamines, tissue was homogenized in 0.1 M perchloric acid and centrifuged at 10,000 rpm for 10 min before filtering through minispin filters for an additional 3 min at 10,000 rpm. The tissue extract were then analyzed by HPLC as described previously (Carta et al., 2007) with minor



**Figure 1.** TH is progressively lost in both the ventral midbrain and striatum of *cNurr1<sup>DATCre</sup>* mice. **A–T**, Confocal microscopy showing TH immunohistochemistry in control (ctrl) and *cNurr1<sup>DATCre</sup>* mice as indicated. **A–J**, Sections were analyzed at the levels of ventral midbrain (as indicated in **A** and **F**) and in the striatum (as indicated in **K** and **P**). TH immunofluorescence was analyzed at both embryonal and postnatal stages as indicated. Results demonstrate a progressive loss of TH immunoreactivity in the ventral midbrain. Note that TH immunoreactivity was more drastically downregulated at more lateral regions compared with the prospective medial VTA. **K–T**, TH immunoreactivity in the striatum. In *cNurr1<sup>DATCre</sup>* mice, TH was lost in the CPu and diminished in the NAc. Scale bars, 250  $\mu$ m.

modifications. Briefly, 25  $\mu$ l of each sample were injected by a cooled autosampler (Midas) into an ESA Coulochem III coupled with an electrochemical detector. The mobile phase (5 g/L sodium acetate, 30 mg/L Na<sub>2</sub> EDTA, 100 mg/L octane sulfonic acid, and 10% methanol, pH 4.2) was delivered at a flow rate of 500  $\mu$ l/min to a reverse phase C18 column (4.6 mm diameter, 150 mm).

**Fluorogold retrograde tracing.** Animals received one unilateral stereotaxic injection in the right striatum using a 5  $\mu$ l Hamilton microsyringe (22 gauge steel cannula) filled with the retrograde tracer Fluorogold (hydroxystilbamidine, 4%, Biotium). The animals were anesthetized with isoflurane, 0.5  $\mu$ l was injected during 1 min, and the cannula was left in place for an additional 2 min before slowly being retracted. The antero-posterior and mediolateral coordinates from bregma were 0.27 and  $-2.10$  mm, respectively, and the dorsoventral coordinates from the dura were  $-2.60$  mm. Animals were killed 4 d after injection, and the brains were isolated. Fresh frozen sections (14  $\mu$ m) were cut with a cryostat and examined under a epifluorescence microscope (Eclipse 11000K; Nikon) coupled to an RTke spot camera.

**Open-field test.** This test was used to monitor overall activity and rearing behavior. The open field consisted of a white plastic box (55  $\times$  35  $\times$  30 cm) with lines (squares of 7  $\times$  35 cm) painted on its floor. The animals were put in the center of the box, habituated for 10 min, and filmed 15 min thereafter while rearing was scored. The video recordings were used to measure the number of lines crossed during the monitoring period. A

line crossing was counted when the mouse moved its whole body from one square to another.

**Stepping test.** Forelimb akinesia was monitored in a modified version of the stepping test, as described previously for rats (Schallert et al., 1992; Kirik et al., 1998). The test was performed three times daily over 3 consecutive days. In this test, the mouse was held firmly by the experimenter with both hindlimbs and one forelimb immobilized, and the mouse was passively moved with the free limb contacting a table surface. The number of adjusting steps, performed by the free forelimb when moved in the forward and backward directions, over a distance of 30 cm, was recorded. Results are presented as data collected on the third testing day.

## Results

### Selective *Nurr1* ablation in late developing mDA neurons

A mouse strain containing a *Nurr1* allele for conditional gene ablation was generated by insertion of two loxP sequences in the second and third introns so that the coding sequence, including the first coding exon 3, is excised by Cre-mediated recombination (supplemental Fig. 1, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). To analyze the consequences of *Nurr1* ablation at late stages of mDA neuron development, we crossed floxed *Nurr1* mice with mice carrying *Cre* inserted in the locus of the *DAT* gene (Ekstrand et al., 2007). Crosses generated *Nurr1* mice that were homozygous for the conditional targeted *Nurr1* allele and heterozygous for the *DAT-Cre* allele (*Nurr1<sup>LoxP/LoxP</sup>;DAT<sup>Cre/wt</sup>*; hereafter referred to as *cNurr1<sup>DATCre</sup>* mice). Littermates of genotype *Nurr1<sup>LoxP/LoxP</sup>;DAT<sup>wt/wt</sup>* or *Nurr1<sup>wt/wt</sup>;DAT<sup>Cre/wt</sup>* were used as controls. Although we cannot ex-

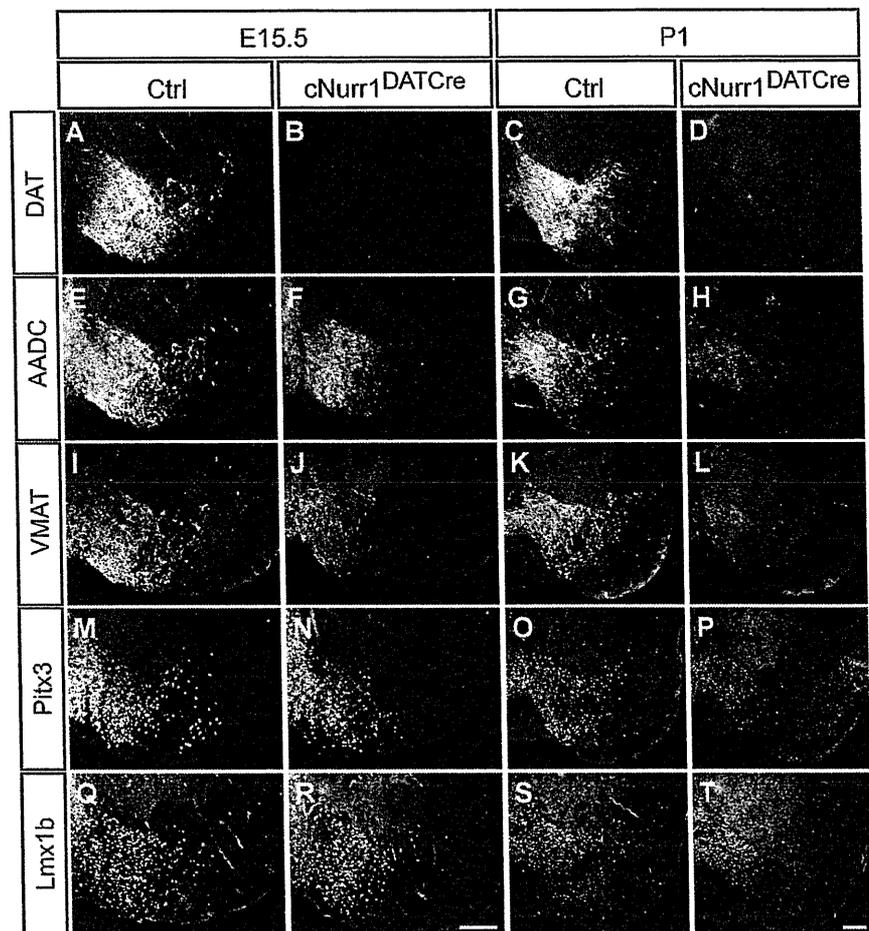
clude that a small number of cells escape *Nurr1* gene deletion, immunohistochemistry using an antibody against *Nurr1* showed that *DAT-Cre*-mediated *Nurr1* ablation resulted in the expected delayed loss of *Nurr1* expression in mDA neurons beginning from approximately E13.5 and becomes essentially complete at E15.5 (supplemental Fig. 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). At this stage of normal development, cells express pan-neuronal properties as well as many mDA neuron markers, and axons are growing toward the developing striatum (Smidt and Burbach, 2007).

*cNurr1<sup>DATCre</sup>* mice were born at the expected Mendelian frequency of  $\sim$ 25% (of a total  $n = 159$ ), however, *cNurr1<sup>DATCre</sup>* mice were less active than controls and did not survive beyond 3 weeks after birth. If litters were allowed to remain with their mothers after weaning, perinatal death was avoided in  $\sim$ 50% of *cNurr1<sup>DATCre</sup>* pups. These surviving mice were, however,  $\sim$ 40% smaller than controls at the age of 2 months (supplemental Fig. 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Although no significant change in spontaneous light-phase locomotor activity could be observed in adult *cNurr1<sup>DATCre</sup>* mice, rearing was dramatically decreased (supplemental Fig. 3, avail-

able at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). L-DOPA treatment of mutant mice did not improve viability and did not induce any weight gain. Instead, *cNurr1<sup>DA1Cre</sup>* mice display a pronounced and severe hypersensitivity to L-DOPA treatment characterized by an acute phase of hyperactivity and repetitive behaviors (including repetitive gnawing, excessive grooming, and self-injury) in all tested mutant ( $n = 9$ ) but not in any wild-type controls ( $n = 7$ ) (see Materials and Methods). These behaviors resemble those that have been observed in neonatal 6-hydroxydopamine lesioned rats treated with L-DOPA (Breese et al., 2005). In conclusion, late embryonic mDA neuron-selective *Nurr1* ablation is associated with decreased weight, rearing, and viability, and mice show an altered response to L-DOPA.

#### Reduced levels of TH and DA in brains of *cNurr1<sup>DA1Cre</sup>* mice

The observed abnormalities are consistent with a dopaminergic deficiency. To analyze the possible cellular basis for the phenotype, brain sections from controls and *cNurr1<sup>DA1Cre</sup>* mice were analyzed by immunohistochemistry using an antibody against TH (Fig. 1). A progressive loss of TH immunostaining in SNc was observed in the *cNurr1<sup>DA1Cre</sup>* mice (Fig. 1*A–J*). TH levels were significantly decreased already at E15.5, soon after *Nurr1* is lost, and decreased further until adulthood when only scattered TH-positive neurons could be detected. TH was diminished also within the VTA at later stages, but a significant number of cells remained even in adult animals (Fig. 1*A–J*). These cells were counted in four non-consecutive sections for each analyzed brain. In adult control VTA, a mean of  $74.5 \pm 7.1$  cells per section were counted in *cNurr1<sup>DA1Cre</sup>* mice ( $n = 4$ ) and  $499.3 \pm 5.2$  cells in controls ( $n = 3$ ) (Student's *t* test,  $4.4 \times 10^{-7}$ ). TH immunostaining within the caudatus putamen (CPU) was completely lost (Fig. 1*K–T*). However, weak immunoreactivity remained in nucleus accumbens (NAc) innervated preferentially by VTA neurons (Fig. 1*K–T*). We also noted the appearance of ectopic TH-positive cell bodies within the striatal parenchyma in *cNurr1<sup>DA1Cre</sup>* mice (supplemental Fig. 4, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). These cells were more frequent in regions in which striatal TH had been most severely depleted as a consequence of *Nurr1* ablation and resemble TH-positive neurons appearing in rodent and primate DA-depletion models (Huo and Parent, 2007). Decreased levels of TH immunostaining were paralleled by decreased DA levels, as measured by HPLC (supplemental Tables 1–3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Striatal DA was dramatically reduced to 14% of controls at P1 in *cNurr1<sup>DA1Cre</sup>* mice and was almost completely lost by P60. An increased ratio of HVA to DA at P14 indicated increased turnover of DA in remaining cells at this stage (supplemental Table 2, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

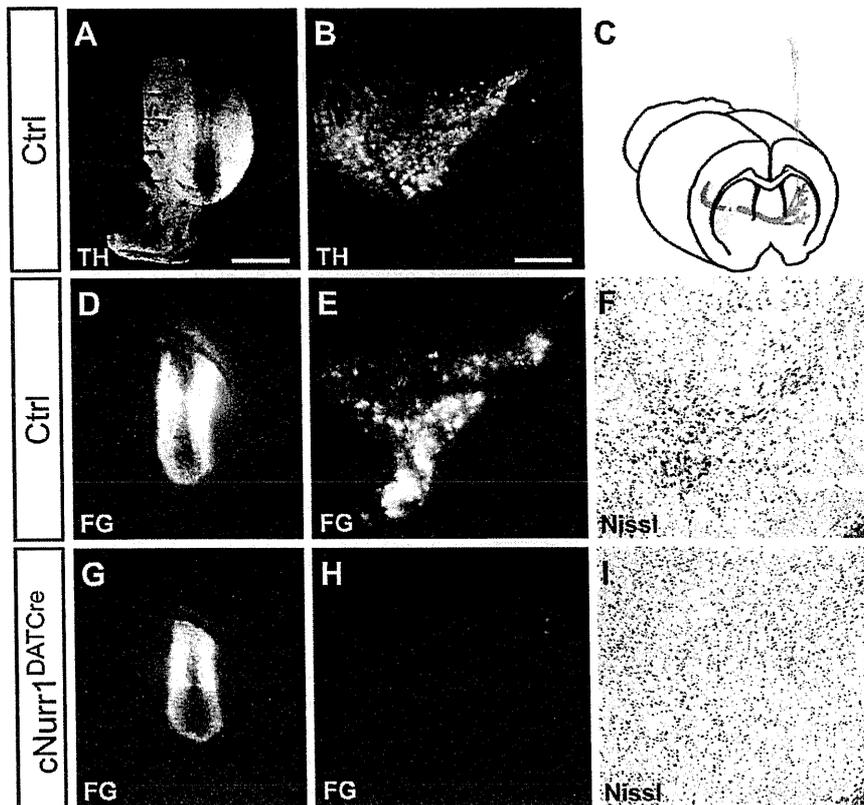


**Figure 2.** All analyzed mDA neuron markers are lost or diminished in *cNurr1<sup>DA1Cre</sup>* mice. *A–T*, Confocal microscopy showing immunohistochemistry of several different mDA neuron markers in control (ctrl) and in *cNurr1<sup>DA1Cre</sup>* mice at E15.5 or at P1, as indicated. The following markers were analyzed: DAT, AADC, VMAT, Pitx3, and Lmx1b. Results show a progressive loss of markers that is more substantial in the lateral SNc, whereas more medial VTA cells are lost more slowly. DAT is completely absent already at E15.5 in *cNurr1<sup>DA1Cre</sup>* mice, whereas all other markers are decreased more slowly in this area (compare *A, B*). Scale bars, 200  $\mu$ m.

rial). DA was more severely decreased in CPU compared with NAc (supplemental Table 3, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). In contrast, 5-HT was significantly increased in both CPU and NAc, consistent with previous findings showing increased serotonergic innervation after striatal DA depletion (Snyder et al., 1986). Thus, a severe neurotransmitter deficiency of the mesostriatal DA system is apparent in *cNurr1<sup>DA1Cre</sup>* mice. Together, measurements of TH immunoreactivity and DA levels demonstrate that *Nurr1* is critically required for maintaining TH expression and DA synthesis from late stages of mDA neuron differentiation.

#### Cellular deficiency within the ventral midbrain of *cNurr1<sup>DA1Cre</sup>* mice

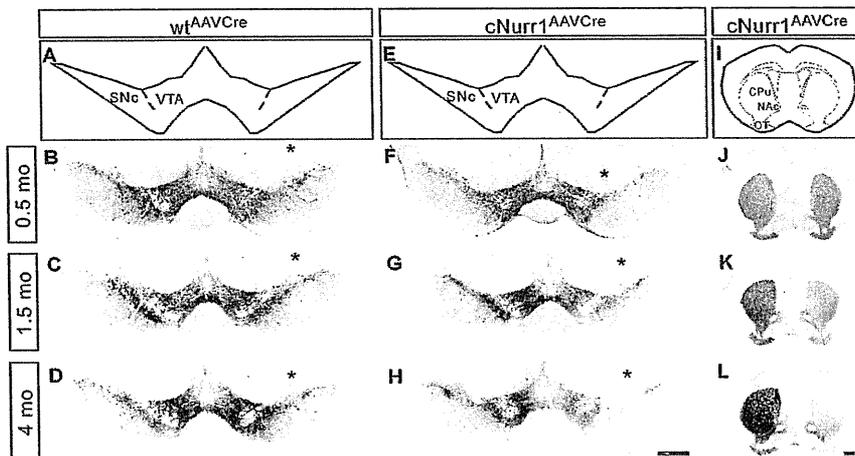
To investigate whether the phenotype is a consequence of a more limited disruption of DA synthesis or a more severe cellular deficiency, a number of additional mDA neuron markers were analyzed. All analyzed mDA neuron markers were diminished or absent within SNc in *cNurr1<sup>DA1Cre</sup>* mice already at E15.5 (Fig. 2). DAT was completely lost at E15.5 and therefore, consistent with previous data (Sacchetti et al., 1999), stands out as being a likely direct target of *Nurr1* (Fig. 2*A–D*). Additional control experiments showed that DAT and other markers, including TH and



**Figure 3.** Cell bodies and striatal innervation are lost in *cNurr1*<sup>DATCre</sup> mice as determined by Fluorogold (FG) retrograde tracing of fibers extending from cell bodies in SNc to the striatum. *A–C*, After Fluorogold injection, injected into the left striatum in either adult (1.5 months old) control (Ctrl) or *cNurr1*<sup>DATCre</sup> mice as indicated in *C*, mice were killed after 4 d and analyzed for TH immunofluorescence in the striatum (*A*) or ventral midbrain (*B*). *D–I*, Analysis for Fluorogold (FG) or by Nissl staining. Strong Fluorogold staining in both striatum (*D*) and in the ventral midbrain (*E*) was consistently seen in all control (Ctrl) animals ( $n = 7$ ). In contrast, Fluorogold fluorescence was only detected in the striatum (*G*) in *cNurr1*<sup>DATCre</sup> mice ( $n = 5$ ), indicating that fibers from the SNc (*H*) had been lost in these animals. Moreover, large, densely packed cell bodies are only visualized by Nissl staining in the ventral midbrain of control animals (*F*) but are completely absent from *cNurr1*<sup>DATCre</sup> mice (*I*). Striatal site of Fluorogold injection is marked by asterisk in *A, D*, and *G*. Scale bars: *A, B, D, E, G, H*, 1  $\mu$ m; *F, I*, 1200  $\mu$ m.

*Nurr1*, were not visibly decreased in mice heterozygous for the *DAT-Cre* allele (supplemental Fig. 5, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (data not shown). In contrast to *DAT*, *AADC*, *VMAT2*, *Pitx3*, or *Lmx1b* were not reduced within the most medial ventral midbrain at this early stage and, with the exception of *DAT*, markers were not completely downregulated at P1 (Fig. 2*E–T*). The progressive loss of markers indicates a severe loss of phenotype within the SNc, whereas cells within the VTA appear more resilient. Importantly, most TH-positive cells within the VTA have lost any detectable expression of *DAT*, indicating that these cells have not escaped *Nurr1* gene targeting (supplemental Fig. 6, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material).

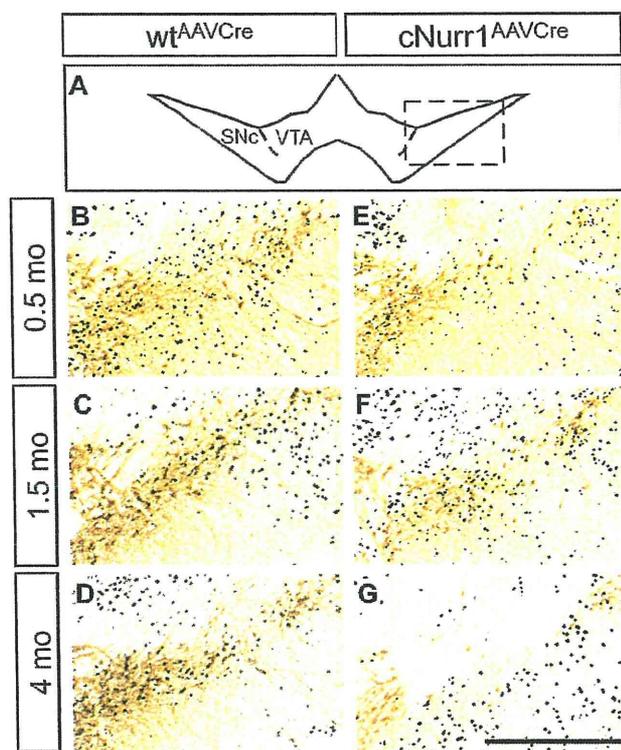
To further assess the extent of a cellular deficiency, striatal target innervation was analyzed by Fluorogold retrograde tracing after injection into the striatum of live 8- to 9-week-old controls and *cNurr1*<sup>DATCre</sup> mice. Fluorogold was transported into SNc cell bodies of control mice; however, fluorescence was entirely undetected within the SNc of Fluorogold-injected *cNurr1*<sup>DATCre</sup> mice (Fig. 3, compare *D, E* with *G, H*). In addition, characteristic large and densely packed TH-immunoreactive mDA neurons within the SNc were virtually absent in *cNurr1*<sup>DATCre</sup> mice (Fig. 3*I*). In conclusion, *Nurr1* ablation in *cNurr1*<sup>DATCre</sup> mice results in rapid loss of SNc cell bodies; however, scattered VTA neurons remained even in adult *cNurr1*<sup>DATCre</sup> mice.



**Figure 4.** TH expression in both the ventral midbrain and striatum is progressively lost in the injected, but not non-injected, side of *cNurr1*<sup>AAVCre</sup> mice. *A–H*, Sections from 0.5, 1.5, and 4 month (mo; as indicated) old AAV-Cre-injected controls (*wt*<sup>AAVCre</sup>) or *cNurr1*<sup>AAVCre</sup> mice were used for analyses by nonfluorescent DAB TH immunostaining in the ventral midbrain. The analyzed region within the ventral midbrain is schematically illustrated in *A* and *E*. The site of injection, marked by an asterisk in *B–D* and *F–H* was verified in all animals by high-power magnification microscopy and was identified as a small area of injection-induced necrosis. Results show that TH immunostaining is not drastically altered at 0.5 months but is progressively decreased at 1.5 and 4 months in the injected SNc and VTA. *I–L*, DAB TH staining at the level of striatum. Analyzed regions are indicated in *I*. TH staining is progressively decreased at 1.5 and 4 months in the side that is ipsilateral to the side of AAV-Cre injection in *cNurr1*<sup>AAVCre</sup> mice (*J–L*). OT, Olfactory tubercle. Scale bars: *A–H*, 600  $\mu$ m; *I–L*, 1 mm.

#### Adeno-associated virus–Cre-mediated *Nurr1* ablation in adult mice

In *cNurr1*<sup>AAVCre</sup> mice, *Nurr1* is ablated well before full mDA neuron maturity and before targets in the striatum have become innervated; thus, it remained possible that the phenotype is a consequence of a developmental dysfunction. Therefore, we proceeded to inactivate *Nurr1* specifically in ventral midbrain of adult mice, using an adeno-associated virus (AAV)–Cre vector driven by the neuron-specific synapsin promoter. AAV-Cre was administered by unilateral stereotaxic microinjection above the right SNc. Cre immunohistochemistry and  $\beta$ -galactosidase expression was analyzed after intranigral AAV-Cre injection into reporter mice in which the ROSA26 locus is targeted with a LacZ reporter gene (Soriano, 1999). Results show widespread Cre expression around the site of injection, spreading into both SNc and VTA, and robust recombination of the LacZ reporter construct (supplemental Fig. 7, available at [www.jneurosci.org](http://www.jneurosci.org) as supple-



**Figure 5.** Cre-expressing cells are lost at 4 months within the SNc after Nurr1 ablation. *A*, Higher magnification showing TH by DAB staining (brown) at the level of the injected SNc in *wtAAVCre* and *cNurr1AAVCre* mice. The region that has been magnified is boxed in *A*. *B–G*, Adjacent sections were immunostained for Cre (black) and are superimposed on the DAB-stained TH sections in all micrographs. Cre staining is widespread in the area in which mDA neurons are normally localized at 0.5 and 1.5 months (*E, F*) but almost completely absent in this area at 4 months (*G*). Scale bar, 500 μm.

mental material). Moreover, except for a small necrotic area around the site of injection, virus administration did not affect tissue morphology, expression of mDA neuron markers, or microglia activation (data not shown).

AAV-Cre was unilaterally injected above the SNc of adult mice homozygous for the floxed *Nurr1* allele to generate adult conditional gene-targeted mice (*cNurr1<sup>AAVCre</sup>*) or into wild-type control mice (*wt<sup>AAVCre</sup>*). In addition, a vector encoding the green fluorescent protein (GFP) driven by the synapsin promoter (AAV-GFP) was injected in mice homozygous for the floxed *Nurr1* allele (*cNurr1<sup>AAV-GFP</sup>* mice) to ensure that the floxed animals are not more sensitive to nonspecific toxicity induced by AAV transduction. Histological analyses were performed from animals killed at 0.5, 1.5, and 4 months after injection.

#### Reduction of TH and DA in adult *Nurr1*-ablated mice

TH immunohistochemistry at the level of the ventral midbrain was analyzed to assess the consequences of adult *Nurr1* ablation. Within SNc, TH immunoreactivity was unaffected at 0.5 months but was progressively reduced at 1.5 and 4 months in the injected SNc in *cNurr1<sup>AAVCre</sup>* mice (Fig. 4*E–H*). In contrast, TH immunoreactivity was unaffected in SNc of control *wt<sup>AAVCre</sup>* and *cNurr1<sup>AAV-GFP</sup>* mice (Fig. 4*A–D*) (data not shown). TH was also reduced in the VTA at 1.5 and 4 months; however, at 4 months, the reduction in VTA was less dramatic compared with SNc (Fig. 4*E–H*).

Decreased striatal TH immunoreactivity paralleled the reduction in the ventral midbrain. Thus, although no signs of degen-

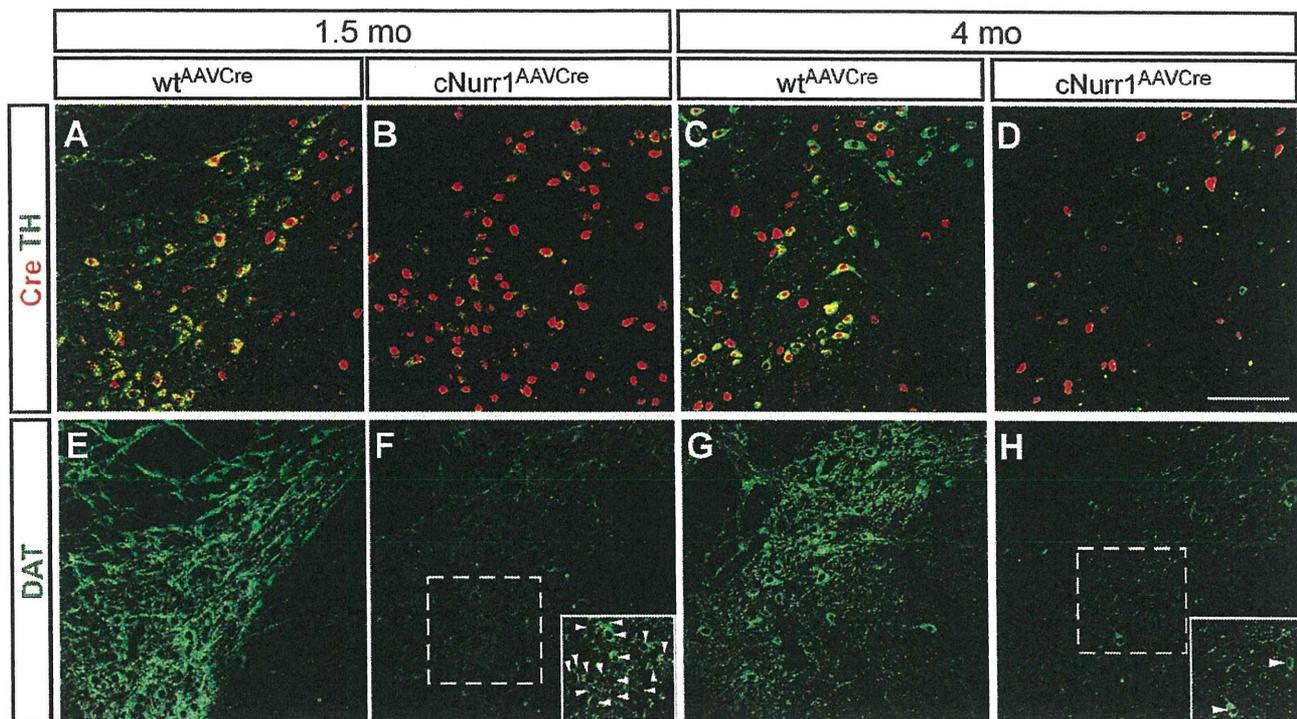
erating striatal TH-stained fibers (swollen axons or dystrophic neurites) were detected, striatal sections ipsilateral to the side of AAV-Cre injection showed clearly reduced TH in *cNurr1<sup>AAVCre</sup>* mice but not in controls (*wt<sup>AAVCre</sup>* or *cNurr1<sup>AAV-GFP</sup>*) (Fig. 4*I–L*) (supplemental Fig. 8, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Diminished TH immunoreactivity was observed in regions innervated by both SNc and VTA (CPu and NAc, respectively), consistent with the reduced TH immunoreactivity in both SNc and VTA mDA neuron cell bodies. Measurement of DA and metabolites by HPLC from dissected tissue at 4 months confirmed this picture as a significant reduction in DA and DA metabolites noted both within the dorsolateral striatum and in areas mostly innervated by the VTA (cortex and ventromedial striatum) (supplemental Table 4, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Thus, TH, DA, and DA metabolites are clearly reduced as a result of adult *Nurr1* ablation.

#### Loss of mDA neuron characteristics in adult *Nurr1*-ablated mice

To further analyze the fate of *Nurr1*-ablated neurons, cells were counted within the SNc and VTA in *cNurr1<sup>AAVCre</sup>* and *wt<sup>AAVCre</sup>* mice. Within SNc, the number of TH-positive cells was significantly decreased at 4 months ( $58.1 \pm 8.3$  and  $95.4 \pm 6.3\%$  in the injected vs non-injected sides of *cNurr1<sup>AAVCre</sup>* and *wt<sup>AAVCre</sup>* mice, respectively;  $p = 0.0053$ ). In contrast, the numbers of TH-positive cells was not significantly reduced within the VTA ( $104.1 \pm 4.7$  and  $101.6 \pm 10.5\%$  in the injected versus non-injected sides of *cNurr1<sup>AAVCre</sup>* and *wt<sup>AAVCre</sup>* mice, respectively). Also, the numbers of TH-positive cells were not significantly changed in *cNurr1<sup>AAVCre</sup>* mice at 1.5 months (data not shown).

To assess the integrity of neurons, cellular analysis was extended by analyzing Cre-immunolabeled sections that were superimposed on adjacent TH-labeled sections (Fig. 5). Notably, in *cNurr1<sup>AAVCre</sup>* mice, Cre expression was clearly detected within the area of SNc at both 0.5 and 1.5 months but was lost at 4 months in the region in which mDA neurons should normally be localized (Fig. 5, compare *E–G* with *B–D*). Cre expression is driven by a general neuronal promoter (synapsin), suggesting that loss of Nurr1 may eventually affect some pan-neuronal properties at 4 months after ablation.

Confocal microscopy confirmed the loss of TH at 1.5 and 4 months after Nurr1 ablation and the loss of Cre at 4 months (Fig. 6*A–D*). At 1.5 months, DAT expression was weak but cell bodies were readily identified (Fig. 6*E, F* and inset in *F*). DAT staining remained also at 4 months, but, at this stage, high-power magnification indicated that some of the staining appeared confined to fibers and/or dystrophic cells (Fig. 6*G, H* and inset in *H*). Nonetheless, at 4 months, most cells with decreased TH stained positive for AADC, showing that not all mDA neuron characteristics were affected (Fig. 7*A–I*). Moreover, VMAT2 is yet another marker that was severely decreased in *cNurr1<sup>AAVCre</sup>* mice, but remaining weakly stained cells were positive for the general neuronal marker Hu (Fig. 7*G–I*). The observed changes were not correlated to increased number of apoptotic cells because increased activated Caspase 3 could not be detected (data not shown). Also, we found no evidence for nigral inflammation or  $\alpha$ -synucleinopathy because activated microglia and  $\alpha$ -synuclein-rich inclusions were not detected at any stage after Nurr1 ablation in *cNurr1<sup>AAVCre</sup>* mice (data not shown). Finally, TH and DAT expression in VTA was also affected, without any apparent loss of the neuronal marker Hu or any signs of dystrophic cells (supplemental Fig. 9, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material) (data not shown). Thus, Nurr1 ablation results in a



**Figure 6.** Decreased expression of DAT and signs of dystrophic cells in *cNurr1<sup>AAVCre</sup>* mice. **A–H**, Confocal analysis of SNc in *wt<sup>AAVCre</sup>* and *cNurr1<sup>AAVCre</sup>* mice at 1.5 and 4 months, as indicated. Confocal images show double staining of Cre (red) and TH (green; **A–D**) and staining for DAT (green; **E–H**). Micrographs show that there is a loss of TH and DAT and a progressive loss of synapsin-driven Cre at 4 months. At 4 months, DAT staining appears fragmented and stains scattered fibers, whereas very few intact cell profiles (marked with arrowheads in **F** and **H**) can be identified in *cNurr1<sup>AAVCre</sup>* mice (compare insets in **F**, **H**). Scale bar, 200  $\mu$ m.

progressive dysfunction characterized by a partial loss of the mDA neuron phenotype. Although we see few signs of neuronal degeneration, we cannot exclude a limited cell loss.

To assess whether the observed dysfunction was paralleled by an altered motor behavior, *cNurr1<sup>AAVCre</sup>* mice were subjected to a stepping test at 3 and 4 months (Schallert et al., 1992; Kirik et al., 1998). Performance of the left forelimb (i.e., the limb contralateral to the vector injection) was impaired at both time points (Fig. 8). Additional behavioral testing, including amphetamine-induced rotations and a corridor test, indicated that individual mutant animals appeared affected; however, the *Nurr1*-ablated group did not show alterations that were statistically significant (supplemental Fig. 10, available at [www.jneurosci.org](http://www.jneurosci.org) as supplemental material). Our results demonstrate progressive mDA neuron dysfunction, leading to a more severe deficiency at 3–4 months after *Nurr1* ablation.

## Discussion

This study provides definitive evidence that *Nurr1* is not only critical for early differentiation but also for the maintenance of functional mDA neurons. Conditional gene targeting at late embryogenesis, when characteristic features of mDA neurons are already apparent, results in a rapid and close to complete mDA neuron loss. Only few TH-positive cells remain within the VTA also in the absence of *Nurr1*. Removal of *Nurr1* leads to a severe dysfunction also in adult mDA neurons. It should be noted that reduction of striatal DA and the behavioral effects after adult ablation most likely underestimate the importance of *Nurr1* in the adult brain because AAV injection only transduced a proportion of all mDA neurons in the injected side of treated animals. Thus, these data emphasize the importance of studying developmental mechanisms for elucidating neuron maintenance mechanisms. An analo-

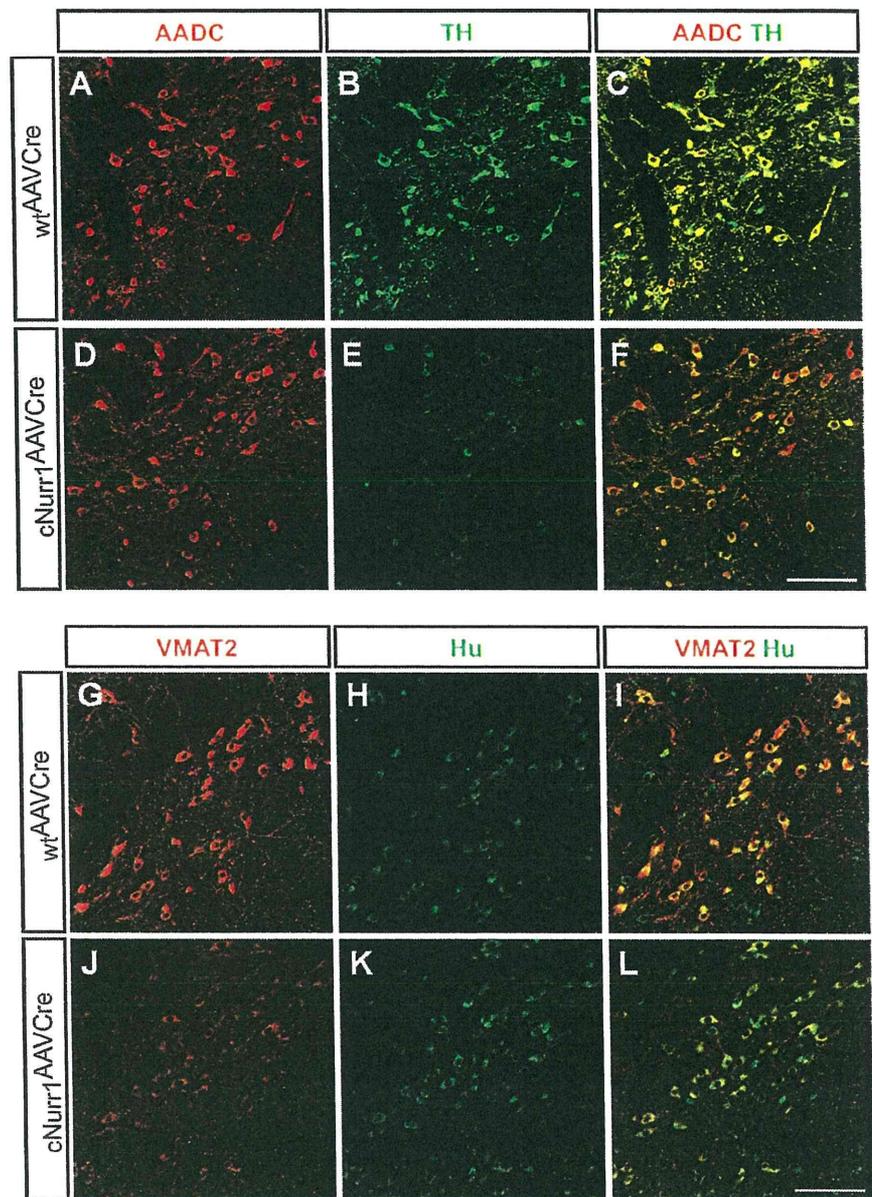
gous example is provided by the glial cell line-derived neurotrophic factor (GDNF). GDNF is known to promote neuronal survival under development, but only recently has conditional gene targeting enabled studies that interrogate the role of GDNF and other factors signaling via Ret for maintenance of midbrain dopamine neurons in the adult brain (Oo et al., 2003; Jain et al., 2006; Kramer et al., 2007; Pascual et al., 2008).

Data presented here have implications for our understanding of how mature differentiated cell types are maintained. Previous studies have indicated that the differentiated state is not irreversible because even mature specialized cells, including for example, olfactory neurons and mature T- and B-cells, can be reprogrammed into undifferentiated pluripotent cells by either somatic cell nuclear transfer or using the recently developed methodology for the generation of induced pluripotent stem cells (Takahashi and Yamanaka, 2006; Gurdon and Melton, 2008). Nevertheless, under normal nonmanipulated conditions *in vivo*, differentiated cells are remarkably stable, indicating the importance of mechanisms that maintain cells in their appropriate differentiated state. Gene targeting in non-neural cell types has revealed how transcription factors functioning in development can be important for the maintenance of terminally differentiated cell types, e.g., Pax5 in B-lymphocytes and Prox1 in lymphatic endothelial cells (Cobaleda et al., 2007; Johnson et al., 2008). In CNS, transcription factors identified for their key roles in early neuron development often continue to be expressed in the adult brain and may therefore guard against loss of phenotype or drift into alternative states (Smidt et al., 1997, 2000; Zetterström et al., 1997; Hendricks et al., 1999; Vult von Steyern et al., 1999; Alberi et al., 2004; Simon et al., 2004; Kang et al., 2007; Kittappa et al., 2007; Smidt and Burbach, 2007; Alavian et al.,

2008). However, remarkably little is known of how these factors function at late stages of development or in the adult. Although examples of adult mDA neuron loss has been reported in mice haploinsufficient for transcription factor genes such as *Engrailed* and *FoxA2*, it remains possible that defects originate during embryonic development (Albéri et al., 2004; Zhao et al., 2006; Kittappa et al., 2007; Sonnier et al., 2007). Importantly, *FoxA2* and *Engrailed* are critical for the establishment of the floor plate and for early midbrain/hindbrain development, respectively, and they are directly and indirectly affecting many cell fates along the entire neuraxis. Thus, haploinsufficiency may cause embryonal deficiencies that do not become manifest until adult stages, a possibility that emphasizes the importance of temporally controlled conditional gene targeting to rigorously test how transcription factors function in terminally differentiated neurons.

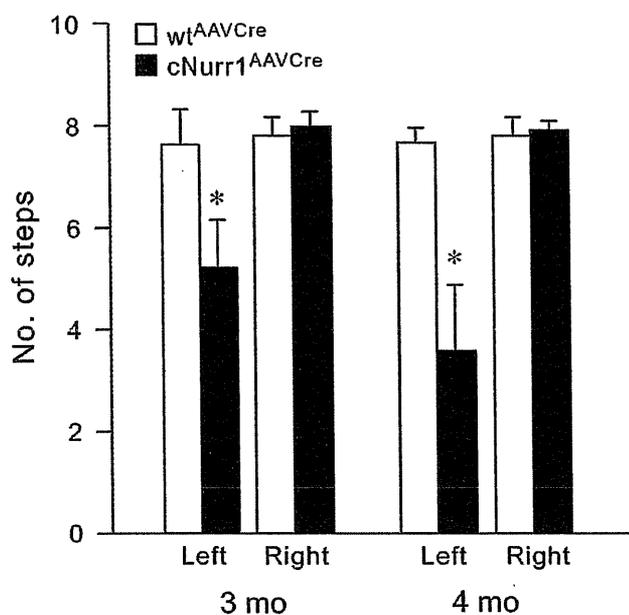
We do not yet understand why *Nurr1* is required in already differentiated mDA neurons. However, data presented here provide compelling evidence for the existence of “terminal selector genes” in mammalian CNS development. Such genes, defined from studies of *Caenorhabditis elegans* neuronal development, are continuously expressed throughout the life of neurons and are essential for both the establishment and maintenance of distinct neuronal phenotypes (Hobert, 2008). Thus, *Nurr1*, which probably regulates typical mDA neuron markers such as *TH*, *DAT*, *AADC*, and *VMAT2* (Sakurada et al., 1999; Sacchetti et al., 2001; Hermanson et al., 2003; Kim et al., 2003), is likely required for both early differentiation and maintenance by regulating genes that distinguish mDA neurons from other neuron types. Presumably, such regulation is critical throughout the life of mDA neurons and would depend on additional components, such as *Pitx3*, in a core transcription factor network (Jacobs et al., 2009).

How may dysregulated *Nurr1* activity contribute to PD? Studies in PD patients have shown that, in early stages of the disease, SNc cell bodies are relatively spared compared with the loss of DA in the putamen (Fearnley and Lees, 1991) and that a significant fraction of the surviving, pigmented, DA somata in the SNc have much reduced expression of the TH enzyme (Hirsch et al., 1988; Chu et al., 2006). This suggests that, during early stages of disease, nigral DA neurons may survive in a dysfunctional state characterized by a downregulated neurotransmitter machinery. An interesting possibility supported by our data is that reduced expression of *Nurr1* contributes to such symptoms. Indeed, *Nurr1* is severely reduced in neurons with signs of pathology in



**Figure 7.** Decreased levels of VMAT2 but not AADC in *cNurr1*<sup>fl/wt</sup> mice. Confocal analysis of SNc in *wt*<sup>AAVCre</sup> and *cNurr1*<sup>fl/wt</sup> mice at 4 months, as indicated. *A–F*, Confocal images show staining of AADC (red; *A, D*), TH (green; *B, E*), and double staining of both markers (*C, F*). Micrographs show that AADC expression appears expressed at normal levels in most cells with decreased levels of TH. *G–L*, Confocal images show staining for VMAT2 (red; *G, J*), Hu (*H, K*), and double staining of both markers (*I, L*). Micrographs show that VMAT2 is severely decreased in *cNurr1*<sup>fl/wt</sup> mice, whereas Hu is maintained at normal levels in essentially all cells with decreased VMAT2. Scale bar, 200  $\mu$ m.

PD brain tissue, and reduced *Nurr1* expression in patients' peripheral blood lymphocytes indicates that diminished *Nurr1* activity may be a systemic feature of disease (Chu et al., 2006; Le et al., 2008). Although such correlations do not determine whether reduced *Nurr1* expression is a cause or a consequence of disease, progressive cell dysfunction in *Nurr1*-ablated mice provides a clear indication that diminished *Nurr1* expression in PD should have deleterious consequences for patients. This view is supported by the identification of *Nurr1* gene variants that have been associated with rare cases of familial and sporadic PD (Xu et al., 2002; Le et al., 2003; Zheng et al., 2003; Jankovic et al., 2005; Grimes et al., 2006; Jacobsen et al., 2008). Although other studies have failed to identify such mutations and indicated that *Nurr1*



**Figure 8.** Forelimb akinesia in the stepping test. The performance of the left paw (contralateral to the vector injection) was significantly impaired in the *fl/fl* mice ( $n = 16$ ) but not the wild-type mice ( $n = 13$ ). Although the impairment was significant at both time points, 3 and 4 months after vector injection, their performance got significantly worse over time ( $p < 0.01$ , Student's paired *t* test): 15 of the 16 mice in the *fl/fl* group showed a decline in their stepping scores between the two tests, and at 4 months, 14 of the *fl/fl* mice had scores below 5 compared with 6 in the 3 month test. Scores give the means of steps recorded in the forehand and backhand direction for each paw (see Materials and Methods). \* $p < 0.001$ , Student's paired *t* test.

gene variants as a cause of PD must be very rare, the combined data from genetics, postmortem PD brain tissue analyses, and the ablation experiments reported here strongly imply that, if and when Nurr1 function is reduced, it will exaggerate PD progression and severity (Wellenbrock et al., 2003; Hering et al., 2004; Tan et al., 2004).

The results indicate that therapies that can restore Nurr1 activity in diseased but not yet degenerated mDA neurons could be of clinical relevance. We envision several strategies whereby Nurr1 activity could be increased. (1) Nurr1 belongs to the nuclear receptor family whose members are commonly regulated by small lipophilic ligands. The putative ligand binding domain of Nurr1 is unconventional and lacks a ligand-binding pocket, but Nurr1 forms heterodimers with retinoid X receptors (RXRs), and ligands activating these receptors can protect mDA neurons in culture (Wallen-Mackenzie et al., 2003; Wang et al., 2003). Thus, RXR may be a relevant target for ligand modulation of Nurr1-regulated processes. It will be important to investigate to what extent Nurr1–RXR heterodimers versus Nurr1 alone are important in pathways associated with the phenotype described in this paper. (2) Nurr1 activity is possible to modulate, for example, by the leukemia drug 6-mercaptopurine (Ordentlich et al., 2003). Although such drugs are pleiotropic and serious side effects are likely, other compounds with higher specificity may be possible to identify. (3) Therapies directed at increasing Nurr1 activity would be effective only as long as sufficient levels of Nurr1 are expressed in diseased neurons. Thus, treatments aimed at restoration of Nurr1 expression by gene delivery may prove advantageous. Using similar AAV vectors as administered in this study may be of particular interest because they have properties that are clinically favorable and are already used in clinical trials in PD patients (Check, 2007).

In conclusion, loss of Nurr1 at stages when characteristic features of mDA neurons are already apparent in the developing embryo or in fully differentiated adult neurons results in loss of mDA neuron-specific gene expression and neuron degeneration. These findings highlight the importance of developmental mechanisms also in the adult brain and clearly indicate that they may be critical for the understanding of cell maintenance and neurodegeneration. How Nurr1 and other transcription factors operate in adult neurons to control and prevent loss or drift in phenotype remains a challenge for future studies.

## References

- Alavian KN, Scholz C, Simon HH (2008) Transcriptional regulation of mesencephalic dopaminergic neurons: the full circle of life and death. *Mov Disord* 23:319–328.
- Albéri L, Scadó P, Simon HH (2004) Engrailed genes are cell-autonomously required to prevent apoptosis in mesencephalic dopaminergic neurons. *Development* 131:3229–3236.
- Andersson E, Tryggvason U, Deng Q, Trifling S, Alekseenko Z, Robert B, Perlmann T, Ericson J (2006) Identification of intrinsic determinants of midbrain dopamine neurons. *Cell* 124:393–405.
- Breese GR, Knapp DJ, Criswell HE, Moy SS, Papadeas ST, Blake BL (2005) The neonate 6-hydroxydopamine lesioned rat: a model for clinical neuroscience and neurobiological principles. *Brain Res Brain Res Rev* 48:57–73.
- Castillo SO, Baffi JS, Palkovits M, Goldstein DS, Kopin H, Witta J, Magnusen MA, Nikodem VM (1998) Dopamine biosynthesis is selectively abolished in substantia nigra ventral tegmental area but not in hypothalamic neurons in mice with targeted disruption of the Nurr1 gene. *Mol Cell Neurosci* 11:36–46.
- Check E (2007) Second chance. *Nat Med* 13:770–771.
- Chu Y, Le W, Kompoliti K, Jankovic J, Mufson EJ, Kordower JH (2006) Nurr1 in Parkinson's disease and related disorders. *J Comp Neurol* 494:495–514.
- Cobaleda C, Jochum W, Busslinger M (2007) Conversion of mature B cells into T cells by dedifferentiation to uncommitted progenitors. *Nature* 449:473–477.
- Eklund AH, Ferzioglu M, Galter D, Zhu S, Hofstetter C, Lindqvist E, Thoms S, Bergstrand A, Hansson PS, Trifunovic A, Hoffer B, Cullheim S, Mohammed AH, Olson L, Larsson NG (2007) Progressive parkinsonism in mice with respiratory chain deficient dopamine neurons. *Proc Natl Acad Sci U S A* 104:1325–1330.
- Fearnley JM, Lees AJ (1991) Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain* 114:2283–2301.
- Grimes DA, Han F, Panisset M, Racacho L, Xiao F, Zou R, Westfall K, Bulman DE (2006) Translated mutation in the Nurr1 gene as a cause for Parkinson's disease. *Mov Disord* 21:906–909.
- Gurdon JB, Melton DA (2008) Nuclear reprogramming in cells. *Science* 322:1811–1815.
- Hendricks T, Francis N, Tyodorov D, Deneris ES (1999) The ETS domain factor Pet 1 is an early and precise marker of central serotonergic neurons and interacts with a conserved element in serotonergic genes. *J Neurosci* 19:10348–10356.
- Hering R, Petrovic S, Mietz EM, Holzmann C, Berg D, Bauer P, Weitzalla D, Müller T, Berger K, Krüger R, Riess O (2004) Extended mutation analysis and association studies of Nurr1 (NR4A2) in Parkinson disease. *Neurology* 62:1231–1232.
- Hermanson E, Joseph B, Castro D, Lindqvist F, Aarnisalo P, Wallen A, Benoit G, Hengeler B, Olson L, Perlmann T (2003) Nurr1 regulates dopamine synthesis and storage in M59D dopamine cells. *Exp Cell Res* 288:324–334.
- Hirsch E, Graybiel AM, Agid YA (1988) Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature* 334:345–348.
- Robert O (2008) Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. *Proc Natl Acad Sci U S A* 105:20067–20071.
- Huo P, Parent A (2007) Dopaminergic neurons intrinsic to the striatum. *J Neurochem* 101:1411–1447.
- Jacobus JM, van Erp S, van der Linden AJ, van Oerthel L, Purbach JP, Smith MP (2009) Pitx3 potentiates Nurr1 in dopamine neuron terminal dif-

- ferentiation through release of SMRT-mediated repression. *Development* 136:531–540.
- Jacobsen KK, MacDonald JJ, Lemonde S, Daigle M, Grimes DA, Bulman DE, Albert PR (2008) A Nurr1 point mutant, implicated in Parkinson's disease, uncouples ERK1/2 dependent regulation of tyrosine hydroxylase transcription. *Neurobiol Dis* 29:117–122.
- Jain S, Golden JP, Wozniak D, Pehek E, Johnson EM Jr, Milbrandt J (2006) RET is dispensable for maintenance of midbrain dopaminergic neurons in adult mice. *J Neurosci* 26:11230–11238.
- Jankovic J, Chen S, Le WD (2005) The role of Nurr1 in the development of dopaminergic neurons and Parkinson's disease. *Prog Neurobiol* 77:128–138.
- Johnson NC, Dillard ME, Baluk P, McDonald DM, Harvey NL, Frase SL, Oliver G (2008) Lymphatic endothelial cell identity is reversible and its maintenance requires Prox1 activity. *Genes Dev* 22:3282–3291.
- Kang BJ, Chang DA, Mackay DD, West GH, Moreira TS, Takakura AC, Gwilt JN, Guyenet PG, Stormetta RL (2007) Central nervous system distribution of the transcription factor Phox2b in the adult rat. *J Comp Neurol* 503:627–641.
- Kim KS, Kim CH, Hwang DY, Seo H, Chung S, Hong SI, Lim IK, Anderson T, Isacson O (2003) Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner. *J Neurochem* 85:622–634.
- Kirik D, Rosenblad C, Björklund A (1998) Characterization of behavioral and neurodegenerative changes following partial lesions of the nigrostriatal dopamine system induced by intrastriatal 6-hydroxydopamine in the rat. *Exp Neurol* 152:259–277.
- Kittappa R, Chang WW, Awatramani RB, McKay RD (2007) The foxa2 gene controls the birth and spontaneous degeneration of dopamine neurons in old age. *PLoS Biol* 5:e325.
- Kramer FR, Aron L, Ramakers GM, Seitz S, Zhuang X, Beyer K, Smidt MP, Klein R (2007) Absence of Ret signaling in mice causes progressive and late degeneration of the nigrostriatal system. *PLoS Biol* 5:e39.
- Le W, Pan T, Huang M, Xu P, Xie W, Zhu W, Zhang X, Deng H, Jankovic J (2008) Decreased NURR1 gene expression in patients with Parkinson's disease. *J Neurol Sci* 273:29–33.
- Le WD, Xu P, Jankovic J, Jiang H, Appel SH, Smith RG, Vassilatis DK (2003) Mutations in NR4A2 associated with familial Parkinson disease. *Nat Genet* 33:85–89.
- Oo TF, Kholodilov N, Burke RE (2003) Regulation of natural cell death in dopaminergic neurons of the substantia nigra by striatal glial cell line derived neurotrophic factor *in vivo*. *J Neurosci* 23:5141–5148.
- Orendtlich P, Yan Y, Zhou S, Heyman RA (2003) Identification of the anti-neoplastic agent 6-mercaptopurine as an activator of the orphan nuclear hormone receptor Nurr1. *J Biol Chem* 278:24791–24799.
- Pascual A, Hidalgo Figueroa M, Pirnat H, Pintado CO, Gómez-Díaz R, López Barneo I (2008) Absolute requirement of GDNF for adult catecholaminergic neuron survival. *Nat Neurosci* 11:755–761.
- Perlmann T, Wallén Mackenzie A (2001) Nurr1, an orphan nuclear receptor with essential functions in developing dopamine cells. *Cell Tissue Res* 318:45–52.
- Sacchetti P, Browschickle LA, Granneman JG, Bannon MJ (1999) Characterization of the 5' flanking region of the human dopamine transporter gene. *Brain Res Mol Brain Res* 74:167–174.
- Sacchetti P, Mitchell TR, Granneman JG, Bannon MJ (2001) Nurr1 enhances transcription of the human dopamine transporter gene through a novel mechanism. *J Neurochem* 76:1565–1572.
- Sakurada K, Ohshima-Sakurada M, Palmer TD, Gage FH (1999) Nurr1, an orphan nuclear receptor, is a transcriptional activator of endogenous tyrosine hydroxylase in neural progenitor cells derived from the adult brain. *Development* 126:4017–4026.
- Sancedo-Cardenas O, Quintana-Hau JD, Le WD, Smidt MP, Cox JJ, De Mayo F, Burbach JP, Comely DM (1993) Nurr1 is essential for the induction of the dopaminergic phenotype and the survival of ventral mesencephalic late dopaminergic precursor neurons. *Proc Natl Acad Sci U S A* 95:4013–4018.
- Schallert T, Norton D, Jones TA (1992) A clinically relevant unilateral rat model of Parkinsonian akinesia. *J Neural Transpl Plast* 3:332–333.
- Simon HH, Thuret S, Alberi L (2004) Midbrain dopaminergic neurons: control of their cell fate by the engrailed transcription factors. *Cell Tissue Res* 318:53–61.
- Smidt MP, Burbach JP (2007) How to make a mesodiencephalic dopaminergic neuron. *Nat Rev Neurosci* 8:21–32.
- Smidt MP, van Schaick HS, Lanctot C, Tremblay JJ, Cox JJ, van der Kleij AA, Wolterink G, Drouin I, Burbach JP (1997) A homeodomain gene Ptx3 has highly restricted brain expression in mesencephalic dopaminergic neurons. *Proc Natl Acad Sci U S A* 94:13305–13310.
- Smidt MP, Asbreuk CH, Cox JJ, Chen H, Johnson RL, Burbach JP (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. *Nat Neurosci* 3:337–341.
- Smidt MP, Smits SM, Burbach JP (2001) Homeobox gene Ptx3 and its role in the development of dopamine neurons of the substantia nigra. *Cell Tissue Res* 318:35–43.
- Snyder AM, Zigmund MJ, Lund RD (1986) Sprouting of serotonergic afferents into striatum after dopamine-depleting lesions in infant rats: a retrograde transport and immunocytochemical study. *J Comp Neurol* 245:274–281.
- Somnier L, Le Pen G, Hartmann A, Bizot JC, Trovero F, Krebs MC, Prochiantz A (2007) Progressive loss of dopaminergic neurons in the ventral mid-brain of adult mice heterozygote for Engrailed 1. *J Neurosci* 27:1063–1071.
- Soriano P (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21:70–71.
- Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126:663–676.
- Tan EK, Chung H, Chandran VR, Tan C, Shen H, Yew K, Pavanni R, Puvan KA, Wong MC, Teoh ML, Yih Y, Zhao Y (2004) Nurr1 mutational screen in Parkinson's disease. *Mov Disord* 19:1503–1505.
- Vult von Steyern P, Martinov V, Rabben I, Nija A, de Lapeyrière O, Lomo T (1999) The homeodomain transcription factors Islet 1 and HB9 are expressed in adult alpha and gamma motoneurons identified by selective retrograde tracing. *Eur J Neurosci* 11:2093–2102.
- Wallen Mackenzie A, Mata de Urquiza A, Petersson S, Rodriguez FI, Friling S, Wagner J, Orendtlich P, Lengqvist J, Heyman RA, Arenas E, Perlmann T (2003) Nurr1 RXR heterodimers mediate RXR ligand induced signaling in neuronal cells. *Genes Dev* 17:3036–3047.
- Wang Z, Benoit G, Liu J, Prasad S, Aarnisalo P, Liu X, Xu H, Walker NP, Perlmann T (2003) Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* 423:555–560.
- Wellenbrock C, Hedrich K, Schäfer N, Kasten M, Jacobs H, Schwinger E, Hagenah J, Pramstaller PP, Vieregge P, Klein C (2003) NR1A2 mutations are rare among European patients with familial Parkinson's disease. *Ann Neurol* 54:415.
- Xu PY, Liang R, Jankovic J, Hunter C, Zeng YX, Ashizawa T, Lai D, Le WD (2002) Association of homozygous 7048G/7049 variant in the intron six of Nurr1 gene with Parkinson's disease. *Neurology* 58:881–884.
- Zetterström RH, Williams R, Perlmann T, Olson L (1996) Cellular expression of the immediate early transcription factors Nurr1 and NGF1 B suggests a gene regulatory role in several brain regions including the nigrostriatal dopamine system. *Brain Res Mol Brain Res* 41:111–120.
- Zetterström RH, Solomin L, Jansson L, Höfler BJ, Olson L, Perlmann T (1997) Dopamine neuron agenesis in Nurr1 deficient mice. *Science* 276:248–250.
- Zhao ZQ, Scott M, Chiechio S, Wang JS, Renner KJ, Gereau RW 4th, Johnson RL, Deneris ES, Chen ZF (2006) Lmx1b is required for maintenance of central serotonergic neurons and mice lacking central serotonergic system exhibit normal locomotor activity. *J Neurosci* 26:12781–12788.
- Zheng K, Heydari B, Simon DK (2003) A common NURR1 polymorphism associated with Parkinson disease and diffuse Lewy body disease. *Arch Neurol* 60:722–725.

医療関係者向け

ALS特集  
Dr.インタビュー1エヒチランスに基づくALSのケア  
前編エヒチランスに基づくALSのケア  
後編学芸情報  
第91回日本神経学会総会  
ランチョンセミナー②

## Dr. インタビュー1



# ALS、その早期診断と QOLの高い患者ケアのために

東京大学医学部附属病院 神経内科 准教授  
郭 伸 先生

&lt;&lt; 1 2 3 4 &gt;&gt;

ALS (Amyotrophic Lateral Sclerosis; 筋萎縮性側索硬化症) は、上位および下位の運動ニューロンがともに選択的に障害される進行性の神経変性疾患である。発症の原因はまだまだ不明の難病で、厚生労働省による特定疾患の1つに指定されている。

ALSは全身の運動ニューロンが障害され得るため、どの部位の運動ニューロンが最初に障害されるかによって初発症状が大きく異なる。そのため、病型によっては早期診断が難しく、神経内科医にたどり着くまでに多くの診療科を経由し、診断までに長期間を要することも少なくない。ALSの診断における問題点と早期診断のためのポイント、さらには診断後のケアについて、東京大学医学部附属病院神経内科の郭先生にお話を伺った。

## ◆ALSは、決して稀な疾患ではない

進行性かつ不可逆性の神経変性疾患であるALSは、極めて稀な疾患であるとよくいわれます。しかし、ALSは他の神経難病と比較して進行が速いため、罹病期間が極めて短いという特徴を勘案すると、一時点の一般人口に占める患者数を表す有病率は極めて低いとしても、一定期間(1年間)における新規発症患者数を表す発症率は決して低くはありません。

ALSの発症についての疫学データをみると、平均発症年齢は50歳代後半で、男性のほうが2~3割多くなっています(図1)。図1で、70歳代以降での発症者数が少ないのは、母集団となる人口での補正を行っていないためであって、実際には、40歳代以降を好発年齢とし、年齢とともに増えていく疾患だと考えるほうが正しいと思います。実際、60歳以降の年間発症率は全人口に対するものの5~10倍に達します。

ALSの発症を考えるうえでの問題の1つは、世界各地における疫学データが、患者数の増加を示していることです。日本でも、特定疾患に指定されているALSの特定疾患医療受給者数は右肩上がりの増加を続けています。発症の原因が解明されていない疾患ですので、患者数増加の正確な理由は知る由もありませんが、人口構成比で補正しても発症率が上昇していることを考えると、高齢化のみでは説明できないと思います。

もう1つの問題は、ALSの平均発症年齢が50歳代の後半にあり、社会的に重要な地位にしている人の多い年代であるということです。これら壮年層が、何ら誘因なく発症して進行性に運動機能を障害するALSに罹患することは、社会的にも大きな損失となります。

ALSの発症原因については、いろいろな可能性が考えられてはいるものの、いまだ特定するには至っていません。ただ、これまでのALSに関する基礎研究は、家族性のALSの一部で認められる、酸化ストレスを解毒する酵素(SOD1)の遺伝子変異によって起こるALSのモデル動物を使って行われてきました。しかし最近では、この家族性ALSと、大多数を占める遺伝子変異のない孤発性のALSとは病態が異なることが明らかになってきたことから、このモデル動物を使った研究成果をそのまま孤発性ALSにあてはめることが問題視されてきています。この反省から、孤発性ALSに見出される分子異常を基にした新たな視点からの研究の重要性が指摘されはじめています。

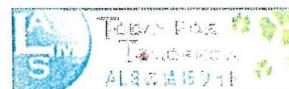
現在、ALSの病因解明の手がかりとして、TDP-43という蛋白の異常な蓄積、およびグルタミン酸システムの過剰な働きによる興奮性神経細胞死という2つのキーワードが浮上ってきて

HOME

ALS特集  
Dr.インタビュー1ALS特集  
Dr.インタビュー2エヒチランスに基づく  
ALSのケア  
前編エヒチランスに基づく  
ALSのケア  
後編学芸情報  
第91回日本神経学会総会  
ランチョンセミナー②

Information

リルテック 50の詳細は  
こちらをご覧ください。

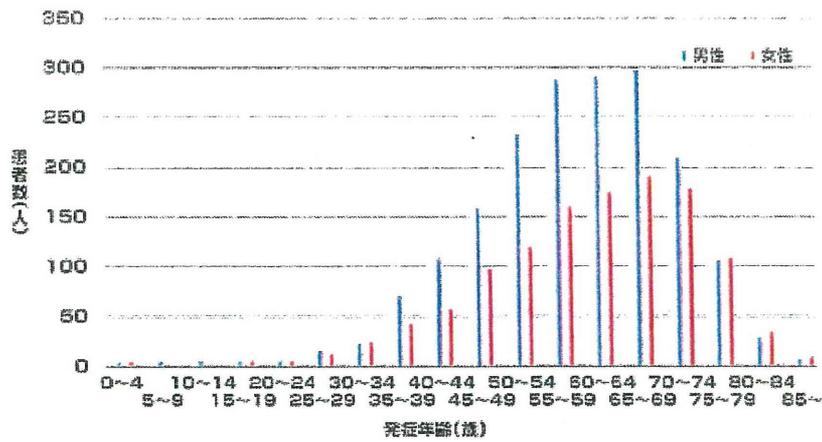


e-MIR  
リルテック50の最新情報はこちら

います。将来的にこの2つを結びつける分子病態が明らかになり、特異的治療法の開発が可能になると、私は期待しています。

図1 日本のALS患者の発症年齢・性別分布(2005)

(特定疾患医療受給者証ベース)



(出典: 永井正規ほか編「電子入力された臨床調査個人票に基づく特定疾患治療研究区審査受給者調査報告書」109, 2005)

<< 1 2 3 4 >>



Copyright © 2006-2009 Nikkei Business Publications, Inc. All Rights Reserved.

日経BP社

医療関係者向け

ALS特集  
Dr.インタビュー2

エヒダンスに基づくALSのケア  
前編

エヒダンスに基づくALSのケア  
後編

学会情報  
日本神経学会総会特別セッション

# Dr. インタビュー1

## ALS、その早期診断と QOLの高い患者ケアのために



東京大学医学部附属病院 神経内科 准教授  
郭 伸 先生

<< 1 2 3 4 >>

### ◆ALSの診断は、除外診断が基本

#### ALSの初発症状

ALSの初発症状は、どの運動ニューロンが侵され、それによりどの筋肉が萎縮し、筋力が低下するかによって大きく異なるため、多彩な症状を示します。ALSは、上位と下位の運動ニューロンがともに変性していく疾患で、ほとんどすべての運動ニューロンと、それに支配される随意筋が障害される可能性があります。唯一の例外が眼球を動かす外眼筋で、多くは末期まで障害されないため、末期のコミュニケーションの手段としても使われています。

図2 上肢の筋萎縮と運動障害

A: 第一指と第二指の間(背側骨間筋)が筋萎縮により陥没している



B: 手内筋の筋萎縮により、手指をまっすぐに伸展できない

具体的には、例えば上肢の遠位が障害されると、前腕筋・手内筋が萎縮します(図2A)。手指の筋力低下のため、患者さんは鉛筆や箸を持つ力がなくなった、ペットボトルが開けられなくなったなどと訴えますし、診察上も手指の伸展障害(図2B)がみられます。同じ上肢の障害であっても、近位の障害が強いと、物をつかむには支障がないものの、肩甲帯の筋萎縮(図3)のため、重い物を高い位置に持ち上げたり、洗髪したりすることが困難になります。

一方、下肢の先端が障害されると、歩いていてつまづきやすくなり、大腿が障害された場合には、階段を上がる、椅子から立ち上がるなどの動作が辛くなります。

舌や咽喉頭の障害のため、構音障害、嚥下障害などが初発症状になることもあります。

図3 肩(三角筋)の筋萎縮によるやせ

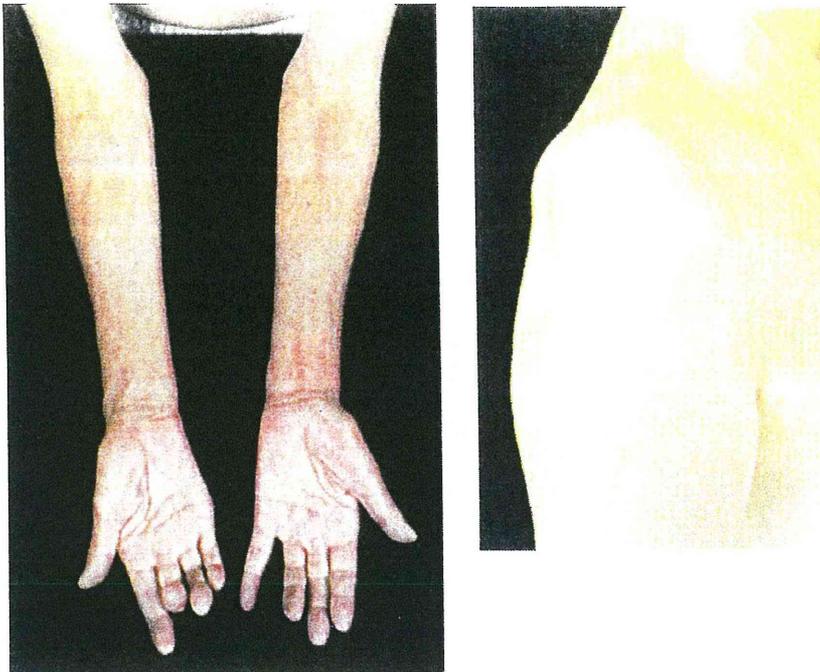
- ▶ HOME
- ▶ ALS特集 Dr.インタビュー1
- ▶ ALS特集 Dr.インタビュー2
- ▶ エヒダンスに基づくALSのケア 前編
- ▶ エヒダンスに基づくALSのケア 後編
- ▶ 学会情報 第51回日本神経学会総会ランチョンセミナー1

#### Information

リルテック50の詳細はこちらをご覧ください。

ALS 50  
Team For  
リルテック50  
ALS患者のサポート

e-MR  
リルテックの製品情報はこちら



#### ALSの診断

ALSには、この検査結果があればALSと診断できるというバイオマーカーがありませんので、同じような症状を呈し得る疾患を鑑別して除外する、除外診断が基本となります。

ALSの診断のためには、上位運動ニューロン障害の徴候(腱反射亢進、病的反射)と、下位運動ニューロン障害の徴候(筋萎縮、筋線維束性攣縮)が、同一の部位で認められることが重要で、ALS診断の1つの決め手となります。筋萎縮が認められる部位での腱反射の亢進は、下位運動ニューロン障害のみられる部位に上位運動ニューロン障害があることを示す所見として重要です。また、針筋電図により下位運動ニューロンの異常を捉えることもできます。ただし、これらの所見がなくても、ALSが否定されるわけではありません。

これら四肢の症状とともに球麻痺がみられれば、ALSに特異性が高く、診断は比較的容易となります。しかし、ALSは初発症状が多彩なうえ、球麻痺のみの場合すらあり、その経過もまた多彩であることが、診断をより難しくしています。少数ではありますが、なかには発症から10年以上が経過しても社会活動を行い得る患者さんもあります。それでも、一貫して症状の進行が認められることは、ALSの診断に重要な要件となりますので、経過を十分に観察することが必要です。

<< 1 2 3 4 >>

日経BP社

© 2006-2009 Nikkei Business Publications, Inc. All Rights Reserved.

日経BP社