Subpopulations of dopaminergic (DA) neurons within the substantia nigra pars compacta (SNpc) display a differential vulnerability to loss in Parkinson’s disease (PD); however, it is not clear why these subsets are preferentially selected in PD-associated neurodegeneration. In rodent SNpc, DA neurons can be divided into two subpopulations based on the expression of aldehyde dehydrogenase 1 (ALDH1A1). Here, we have shown that, in α-synuclein transgenic mice, a murine model of PD-related disease, DA neurodegeneration occurs mainly in a dorsomedial ALDH1A1-negative subpopulation that is also prone to cytotoxic aggregation of α-synuclein. Notably, the topographic ALDH1A1 pattern observed in α-synuclein transgenic mice was conserved in human SNpc. Postmortem evaluation of brains of patients with PD revealed a severe reduction of ALDH1A1 expression and neurodegeneration in the ventral ALDH1A1-positive DA subpopulations. ALDH1A1 expression was also suppressed in α-synuclein transgenic mice. Deletion of Aldh1a1 exacerbated α-synuclein–mediated DA neurodegeneration and α-synuclein aggregation, whereas Aldh1a1-null and control DA neurons were comparably susceptible to 1-methyl-4-phenylpyridinium−, glutamate−, or camptothecin-induced cell death. Aldh1a1 overexpression appeared to preferentially protect against α-synuclein–mediated DA neurodegeneration but did not rescue α-synuclein–induced loss of cortical neurons. Together, our findings suggest that ALDH1A1 protects subpopulations of SNpc DA neurons by preventing the accumulation of dopamine aldehyde intermediates and formation of cytotoxic α-synuclein oligomers.

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research article

...uated the overall degeneration of SNpc DA neurons, whereas overexpression of ALDH1A1 was neuron protective. Finally, we tested the specificity of ALDH1A1 in α-synuclein–induced midbrain DA neuron loss. We found that Aldh1a1−/− and Aldh1a1+/− midbrain DA neurons showed no difference in 1-methyl-4-phenylpyridinium+– (MPP+–), glutamate−, and camptothecin–mediated cell death, whereas overexpression of ALDH1A1 did not rescue the α-synuclein–induced loss of cortical neurons. Our findings demonstrate that ALDH1A1 is an important molecular determinant responsible for the differential susceptibility of SNpc DA neurons in PD and further support the notion that the reactive aldehyde byproducts derived from abnormal cytosolic dopamine oxidation may contribute to the DA neuron loss by facilitating the formation of cytotoxic α-synuclein oligomers (17).

Results

ALDH1A1-negative DA neurons in the dorsal SNpc are preferentially degenerated in A53T transgenic mice. As shown previously (10), a progressive loss of tyrosine hydroxylase–positive (TH-positive) DA neurons was observed in the SNpc of 18-month-old A53T transgenic mice as compared with that in age-matched nontransgenic (nTg) littermate controls (Figure 1, A and B). To determine whether the loss of DA neurons occurs randomly throughout SNpc or shows any regional preference, we calculated the total space occupied by the SNpc DA neurons and measured the shortest distance between neighboring neurons. If neurons died randomly in the SNpc, we expected to observe a significant increase of distance between remaining neurons, while the volume of SNpc might not change substantially. In contrast, we observed more than 40% reduction of SNpc volume but only a mild increase of neuron spacing in the mutant mice (Figure 1, C and D). These data suggest that DA neurons may not degenerate randomly in the SNpc of A53T transgenic mice, implying that regional selectivity of SNpc DA neuron loss in the mutant mice may exist.

To identify the regions that show more substantial loss of DA neurons in the SNpc, we generated 3D reconstructions of DA neurons in the midbrains of 18-month-old A53T and nTg mice. We found the loss of DA neurons mainly occurred in the dorsomedial (DM) tier of SNpc in A53T mice (arrows, Figure 1E and Supplemental Videos 1 and 2; supplemental material available online with this article; doi:10.1172/JCI72176DS1). This observation suggests that DA neurons from the DM and ventrolateral (VL) tiers of SNpc may exhibit differential susceptibility to α-synuclein–induced cytotoxicity.

We then tried to identify the molecular determinants in the VL SNpc DA neurons that are responsible for the increased resistance to α-synuclein–mediated cytotoxicity. A previous study shows that ALDH1A1 is selectively expressed by a subset of DA neurons located predominantly in the VL tier of rodent SNpc (11). We therefore examined the distribution of both TH– and ALDH1A1-positive (TH+/ALDH1A1+) DA neurons in the SNpc of A53T and control mice. Consistent with the previous finding (11), these double-positive neurons were located primarily in the VL tier of SNpc (Figure 1F). At the subcellular level, ALDH1A1 showed a similar cytosolic distribution pattern as TH in DA neurons (Figure 1G). There was a modest but marked loss of TH+/ALDH1A1+ DA neurons in the SNpc of 1-month-old A53T mice; however, no additional loss of these neurons was found in 18-month-old A53T mice (Figure 1, F and H). By contrast, the TH-positive but ALDH1A1-negative (TH+/ALDH1A1−) DA neurons, located mainly in the DM tier of SNpc, displayed remarkably progressive degeneration in the mutant mice from 1 to 18 months of age (arrowheads, Figure 1, F and I). The ALDH1A1-positive and -negative DA neurons also maintained a conserved topographic distribution in the ventral tegmental area (VTA) of midbrain (Supplemental Figure 1). In addition, a similarly selective loss of ALDH1A1-negative DA neurons was found in the DM tier of VTA in 18-month-old A53T mice (Figure 1, J–L). These observations demonstrate the differential susceptibility of ALDH1A1-positive and -negative SNpc DA neurons in response to α-synuclein–mediated genetic insults during the aging process, in which the ALDH1A1-positive DA neurons in the VL tier of SNpc are more resistant to α-synuclein–induced neurodegeneration.

ALDH1A1-negative SNpc DA neurons contain more aggregated α-synuclein. ALDH1A1 regulates the metabolism of dopamine through oxidation of its biogenic aldehyde intermediate, DOPAL (12). DOPAL is a potent cross-linker of α-synuclein, which promotes the polymerization of α-synuclein (12, 13, 18). Consistently, DOPAL increased the oligomerization of recombinant α-synuclein protein in a cell-free assay (Figure 2A). We speculate that ALDH1A1-negative DA neurons may contain higher levels of DOPAL than the ALDH1A1-positive ones, which may lead to a greater accumulation of cytotoxic α-synuclein aggregates in the ALDH1A1-negative DA neurons. In support of this notion, we found substantially more proteinase K–resistant (PK-resistant) α-synuclein aggregates in the soma of ALDH1A1-negative SNpc neurons, although the levels of total transgenic α-synuclein were comparable between ALDH1A1-positive and -negative neurons (Figure 2, B–D). The levels of transgenic α-synuclein and ALDH1A1 expression were also plotted for each neuron under nontreated and PK-treated conditions (Figure 2E). There is no correlation between the levels of transgenic α-synuclein and ALDH1A1 expression in nontreated samples (Spearman correlation test, P = 0.6083). By contrast, a reverse correlation between the levels of aggregated α-synuclein and ALDH1A1 was found in PK-treated samples (P < 0.0001).

We next examined the level of PK-resistant α-synuclein aggregates in the axons of SNpc DA neurons that project to the dorsal striatum. ALDH1A1 and transgenic α-synuclein were extensively colocalized in the midbrain DA axons and axon terminals at the striatum (Figure 2F), confirming a highly selective expression of Aldh1a1 among Aldh family genes in the midbrain DA neurons (Supplemental Figure 2). The total transgenic human α-synuclein was comparable in both ALDH1A1-positive and -negative axon fibers in the nontreated sections, whereas PK-resistant α-synuclein aggregates appeared only in the ALDH1A1-negative fibers (Figure 2F). PK-resistant α-synuclein aggregates have been shown to generate higher levels of oxidative stress and are more toxic to cells (19–21). Therefore, these results suggest that ALDH1A1 may improve the survival of DA neurons via attenuating the formation of PK-resistant α-synuclein aggregates.

ALDH1A1-positive DA neurons exhibit a conserved topographic distribution in human SNpc. To investigate whether ALDH1A1 plays a similar role in defining two anatomically distinct subpopulations of DA neurons in the human SNpc, we stained normal human SNpc sections with antibodies against ALDH1A1. The SNpc DA neurons were identified by the presence of brown-colored neuromelanin (NM) in the soma, while the staining of ALDH1A1 appeared as a purple color present in both soma and neurites (Figure 3A). A clear clustering of ALDH1A1-positive and -negative DA neurons was found in the control human SNpc (Figure 3A). The
Figure 1
ALDH1A1-negative nigrostriatal DA neurons in the dorsal SNpc are preferentially degenerated in A53T transgenic mice. (A) TH staining (brown) of midbrain coronal sections (top, bregma –2.90, bottom, bregma –3.20) of 18-month-old A53T and littermate nTg female mice. (B) Number of TH-positive neurons remaining in SNpc of 1-month-old (n = 3 per genotype) and 18-month-old (n = 5 per genotype) A53T and littermate nTg female mice. (C) Volume of SNpc of 18-month-old A53T and littermate nTg female mice (n = 5 per genotype). (D) Minimal distance between neighboring DA neurons in SNpc of 18-month-old A53T and littermate nTg female mice. 1,336 and 1,060 nigrostriatal DA neurons from 3 pairs of A53T and nTg mice were analyzed, respectively. (E) 3D reconstruction of TH-positive neurons distributed in SNpc, VTA, and retrorubral field (RRF) of 18-month-old A53T and control nTg female mice. Arrows point to DM tier of DA neurons in SNpc of nTg and A53T mice. D, dorsal; L, lateral; Ro, rostral. (F) Representative images show TH and ALDH1A1 staining in midbrain coronal sections (bregma –2.92) of 1- and 18-month-old A53T and 18-month-old nTg female mice. Arrowheads indicate DM ALDH1A1-negative DA neurons. Arrowheads point to the DM tier of SNpc. (G) Representative images show ALDH1A1 and TH staining in DA neurons of 1-month-old A53T nTg female mice. Arrowheads indicate DM ALDH1A1-negative DA neurons. Arrowheads point to the DM tier of SNpc. (H) Numbers of TH-positive/ALDH1A1-positive DA neurons in nTg and A53T mice. (I) TH-positive/ALDH1A1-negative DA neurons remaining in SNpc of 1-month-old (n = 3 per genotype) and 18-month-old (n = 5 per genotype) A53T and nTg female mice. (J–L) Numbers of total (J) TH-positive, (K) TH-positive/ALDH1A1-positive, and (L) TH-positive/ALDH1A1-negative DA neurons remaining in VTA of 1-month-old (n = 3 per genotype) and 18-month-old (n = 5 per genotype) A53T and nTg female mice. Scale bar: 100 μm (A and F); 20 μm (G). (A and F) The dashed line marks the boundary between SNpc and VTA. *P < 0.05, **P < 0.01, ***P < 0.001.
existence of distinguished subpopulations of ALDH1A1-positive and -negative DA neurons in the control human SNpc was further confirmed by immunofluorescence staining with antibodies against ALDH1A1 and TH (Figure 3B). To reveal the topographic distribution of ALDH1A1-positive and -negative DA neurons in the human SNpc, we stained a series of transverse sections evenly sampled from the entire SNpc of 10 sporadic PD and 9 age- and gender-matched control cases (Supplemental Table 1) and generated 3D maps of these two populations of DA neurons along the dorsal-ventral and medial-lateral axis of each SNpc (Figure 3C and Supplemental Figure 3). In all control cases, ALDH1A1-positive DA neurons were concentrated in the ventral tier of SNpc, whereas ALDH1A1-negative DA neurons were primarily located in the dorsal tier (Figure 3C, Table 1, and Supplemental Figure 3A). Therefore, ALDH1A1-positive DA neurons display a topographically conserved distribution pattern in both rodent and human SNpc.

Human SNpc can be divided into 6 morphometric regions: in the dorsal tier, the medial (DM) part, lateral (DL) part, and pars
Figure 3
ALDH1A1-positive DA neurons exhibit a conserved topographic distribution in human SNpc and show reduction of ALDH1A1 expression in PD. (A) Representative bright-field image shows ALDH1A1 staining (purple) in SNpc transverse sections of control human brains. Nigrostriatal DA neurons (dark brown) were marked by the presence of NM in the soma. Arrow points to a cluster of ALDH1A1-negative DA neurons in the dorsal tier of SNpc. Arrowhead indicates a group of ALDH1A1-positive DA neurons in the ventral tier of SNpc. (B) Representative fluorescent images show TH and ALDH1A1 containing in the transverse section of SNpc in control human brains. (C) 3D reconstruction of DA neurons in human SNpc shows the distribution of ALDH1A1 and NM double-positive (purple) as well as ALDH1A1-negative/NM-positive (brown) neurons remaining in the SNpc of control (Ctrl) and PD human brains. (D) Sketch of transverse section of human SNpc. RN, red nucleus. (E–I) Remaining DA neurons in the (E) SNpc, (F) dorsal, (G) PL, (H) VM, and (I) VL parts of control ((n = 9) and PD (n = 10) brains. Numbers over the PD bars show the percentage of DA neuron loss compared with the controls. (J and K) Percentages of ALDH1A1+/NM− and ALDH1A1+/NM+ DA neurons in SNpc, dorsal, PL, VM, and VL parts of (J) control human (n = 9) and (K) PD cases (n = 10). (L) Percentages of ALDH1A1+/NM− and ALDH1A1+/NM+ DA neurons in SNpc, dorsal, PL, VM, and VL parts of control (n = 9) and PD cases (n = 10). (M–P) Total number and percentage of ALDH1A1+ and ALDH1A1−/NM+ neurons in the (M and N) SNpc and (O and P) VL subregion of control, mild, moderate, and severe PD cases. Scale bar: 70 μm (A); 100 μm (B). *P < 0.05, **P < 0.01, ***P < 0.001.

Reduction of ALDH1A1 expression in the ventral ALDH1A1-positive subpopulations of nigrostriatal DA neurons in PD. There was an average of 66% loss of DA neurons in the SNpc of PD brains compared with that in age-matched control cases (Figure 3E). We further estimated the severity of neurodegeneration is comparable between DM and DL in the dorsal tier and within VI and VL in the ventral tier (3), we merged DM and DL as dorsal part and VI and VL as VL part in this study (Figure 3D). We calculated the percentage of DA neurons that remained in each SNpc region of control and PD cases (Figure 3, E–I, and Table 1). Our results indicate that, in normal human SNpc, the dorsal part had more ALDH1A1-negative DA neurons (69%), whereas the VL part contained the majority of ALDH1A1-positive neurons (92%, Table 1 and Figure 3, J and L).

Overexpression of α-synuclein suppresses ALDH1A1 expression in the midbrain DA neurons of A53T transgenic mice. Reduction of ALDH1A1 mRNA expression has also been reported previously in postmortem PD brains (22). We then examined whether the expression of Aldh1a1 mRNA and protein was affected in the SNpc DA neurons of A53T transgenic mice. We used striatal tissues for protein expression analysis instead of midbrain tissues, because the dissection of striatal tissues is more consistent between different animals and the striatal ALDH1A1 and transgenic α-synuclein proteins were derived mainly from the axons and axon terminals of midbrain DA neurons projected to the striatum (ref. 10 and Figure 2E). We collected the striatum from 2-month-old nTg and A53T mice and checked the expression of ALDH1A1, α-synuclein,

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ALDH1A1-negative, A−; ALDH1A1-positive, A+.

Table 1
List of the number and percentage of remaining DA neurons in the SNpc and its subdivisions in control and PD cases

ALDH1A1 expression is reduced in A53T transgenic mice. (A) Western blot shows expression of ALDH1A1 protein in the striatum of 3 independent pairs of 2-month-old A53T transgenic and littermate nTg female mice. (B) The relative expression levels of ALDH1A1 in the striatal homogenate of 2-month-old nTg (n = 3) and A53T (n = 3) female mice. (C) Aldh1a1 mRNA expression in nigrostriatal DA neurons isolated from 2-week-old control and littermate A53T transgenic mice (n > 1,000 cells per genotype). β-Actin was used to normalize the gene expression from different samples. (D) Western blot analysis shows ALDH1A1 expression in striatal tissues of 2-month-old A53T mice compared with that in the control mice (Figure 4, A and B). Therefore, overexpression of α-synuclein in DA neurons may suppress the expression of ALDH1A1 protein in these neurons. We next examined this hypothesis directly by quantifying the expression of Aldh1a1 mRNA in the SNpc DA neurons isolated by laser capture microdissection (LCM) from 2-week-old control and A53T mice. We observed a substantial reduction of Aldh1a1 mRNA expression in the SNpc DA neurons of A53T mice compared with that in the controls (Figure 4C). A similar decrease of Aldh1a1 mRNA expression was also observed in the midbrain tissues of 2-month-old A53T mice (P = 0.0289). We next examined the expression of ALDH1A1 in the striatum of 1-, 6-, and 18-month-old nTg and A53T transgenic mice (Figure 4D and E). Both genotypes (2-way ANOVA, interaction: F1,18 = 130.10, P < 0.0001) and age (interaction: F2,18 = 25.17, P < 0.0001) affect the expression of ALDH1A1; the expression level of ALDH1A1 was substantially decreased in 18-month-old mice compared with that in the 1-month-old mice in both genotypes (Figure 4E). Together, these data suggest that both aging and pathogenic α-synuclein overexpression suppress the expression of ALDH1A1 in midbrain DA neurons.

Genetic deletion of Aldh1a1 exacerbates SNpc DA neurodegeneration and motor behavioral impairments in A53T transgenic mice. To further establish the function of ALDH1A1 in protecting against SNpc DA neuron loss, we crossbred A53T transgenic mice with Aldh1a1−/− mice to generate A53T transgenic mice in both the Aldh1a1-null (A53T/Aldh1a1−/−) and wild-type (A53T/Aldh1a1+/+) background. Aldh1a1−/− mice did not exhibit any loss of SNpc DA neurons at 18 months of age (refs. 16, 24, and Figure 5, A and B). By contrast, A53T/Aldh1a1−/− mice developed more severe loss of SNpc DA neurons compared with control A53T/Aldh1a1+/+ mice (Figure 5, A and B). Similarly, Aldh1a1−/− mice showed no obvious motor behavioral phenotypes in both open-field and rotarod tests (Figure 5, C–E). However, A53T/Aldh1a1−/− mice developed further motor impairments in both of these two tests compared with A53T/Aldh1a1+/+ mice (Figure 5, C–E). Therefore, these in vivo studies provide additional evidence for and support a protective function of ALDH1A1 against α-synuclein–induced SNpc DA neurodegeneration.

Gene silencing of Aldh1a1 reduces the accumulation of cleaved caspase-3, the active form of caspase-3, in TH-positive neurons (Figure 7A). Around 30% to 40% loss of TH-positive neurons was observed from the A53T cultures as...
compared with that from the littermate controls, whereas administration of doxycycline (DOX) that suppresses the expression of α-synuclein transgene prevented the DA neuron loss (ref. 10 and Figure 7, B and C). We found that overexpression of ALDH1A1 significantly increased the survival of DA neurons derived from both A53T and control pups (Figure 7, B and C). Moreover, the presence of extra ALDH1A1 effectively rescued the loss of DA neurons derived from A53T transgenic mice (Figure 7C). In addition, an ALDH1A1 activator, 6-methyl-2-phenylazo-3-pyridinol (SIB1757) (26), also significantly improved the survival of TH-positive neurons in A53T cultures (Figure 7D). Together, these observations provide direct support for a protective function of ALDH1A1 in maintenance of the survival of midbrain DA neurons against α-synuclein–induced cytotoxicity.

**ALDH1A1 preferentially protects against α-synuclein–mediated midbrain DA neuron loss.** To further test a specific role of ALDH1A1 in α-synuclein–induced midbrain DA neuron loss, we treated cultured midbrain DA neurons derived from Aldh1a1–/– and Aldh1a1+/– mice with 3 different types of cell death inducers: MPP⁺, a known mitochondrial complex I inhibitor that causes DA neuron death (27); glutamate, which induces excitotoxicity (28); and camptothecin, a DNA enzyme topoisomerase I inhibitor that causes apoptosis (29). We found that Aldh1a1–/– and Aldh1a1+/– midbrain DA neurons showed comparable susceptibility to MPP⁺-, glutamate-, and camptothecin–induced cell death (Figure 8, A–C). These data provide evidence that ALDH1A1 may preferentially protect against α-synuclein–induced neuron loss.

Next, we examined whether ALDH1A1 protects against α-synuclein–induced neuron loss preferentially in DA neurons. We cultured cortical neurons from CaMKII-tTA/tetO-A53T transgenic mice, which overexpress PD-related A53T α-synuclein in the forebrain neurons and develop extensive loss of cortical neurons (30). We then tested whether ALDH1A1 prevents the α-synuclein–induced loss of cortical neurons by infecting these neurons with ALDH1A1 and control GFP-expressing AAV vectors. We found that overexpression of ALDH1A1 did not improve the survival of cortical neurons compared with the GFP controls (Figure 8D). In contrast, DOX treatment that suppressed the expression of transgenic α-synuclein significantly improved the survival rate of these cortical neurons (Figure 8D and Supplemental Figure 5).

These results suggest a preferential role of ALDH1A1 in protecting α-synuclein–induced DA neuron loss.

**Discussion**

Previous studies reveal some very special characteristics of the SNpc DA neurons, such as having a long, unmyelinated and highly ramified axonal structure; using dopamine as the transmitter; and being pace-making neurons that keep firing through a l-type calcium channel (31). As the result of these characteristics, the high neural activity, the accumulation of biogenic dopamine metabolites and other reactive oxygen species, and the alteration of calcium homeostasis may make the SNpc DA neurons more vulnerable to PD-related degeneration (31–33). In addition, DA neurons residing in different subdivisions of SNpc also display differential vulnerability in PD (2–4). However, the underlying molecular mechanism is unclear. Here, we report that a preferential degeneration of DA neurons also occurred in the DM tier of SNpc in α-synuclein transgenic mice. Furthermore, we have shown that these neurons were primarily ALDH1A1-negative DA neurons, by which ALDH1A1 provides an important molecular marker that divides SNpc DA neurons into two subpopulations corresponding to their differential susceptibility in α-synuclein–induced DA neurodegeneration. Interestingly, ALDH1A1-positive and -negative DA neurons exhibited a very similar topographic distri-

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**Figure 5**

Genetic ablation of ALDH1A1 exacerbates the SNpc DA neurodegeneration and motor behavioral impairments in A53T transgenic mice. (A) Representative images show ALDH1A1 and TH staining in the midbrain coronal sections of 18-month-old Aldh1a1+/–, Aldh1a1–/–, A53T/Aldh1a1+/–, and A53T/Aldh1a1–/– mice. Scale bar: 100 μm. (B) Numbers of all TH-positive neurons remaining in the SNpc of 18-month-old Aldh1a1+/–, Aldh1a1–/–, A53T/Aldh1a1+/–, and A53T/Aldh1a1–/– mice (n = 5 per genotype, 2 males and 3 females per genotype). (C and D) The 6-month-old Aldh1a1+/– (n = 28, 20 males and 8 females), Aldh1a1–/– (n = 16, 11 males and 5 females), A53T/Aldh1a1+/– (n = 31, 22 males and 9 females), and A53T/Aldh1a1–/– (n = 13, 8 males and 5 females) mice were subjected to open-field tests. The (C) ambulatory and (D) rearing activities were quantified. (E) The latency to fall was quantified by the rotarod test in 6-month-old Aldh1a1+/– (n = 23, 18 males and 5 females), Aldh1a1–/– (n = 13, 8 males and 5 females), A53T/Aldh1a1+/– (n = 22, 15 males and 7 females), and A53T/Aldh1a1–/– (n = 12, 8 males and 4 females) mice. *P < 0.05, **P < 0.01.
bution in the SNpc of human brains, i.e., ALDH1A1-positive neurons were mainly located in the ventral tier of the SNpc, whereas ALDH1A1-negative neurons were concentrated in the dorsal tier. An early study shows that those dorsal SNpc DA neurons are more prone to aging-related neuronal loss, while the ventral DA neurons are largely spared in human SNpc (3). In our mouse studies, we found that the expression of ALDH1A1 was decreased in control mice during aging; however, we did not observe any significant loss of SNpc DA neurons in the aged control mice. Furthermore, genetic deletion of Aldh1a1 in mice also did not cause degeneration of SNpc DA neurons in aged Aldh1a1−/− mice. Together, no age-related loss of SNpc DA neurons was found in both control and Aldh1a1−/− mice. By contrast, in our α-synuclein transgenic mice, we found that ALDH1A1-negative but not-positive SNpc DA neu-

Figure 6
Genetic inhibition of Aldh1a1 promotes α-synuclein aggregation in A53T transgenic mice. (A) Human α-synuclein and ALDH1A1 costaining in the nontreated or PK-treated DL striatum sections of 18-month-old A53T/Aldh1a1+/+ and A53T/Aldh1a1−/− mice. Scale bar: 10 μm. (B and C) The average intensity of α-synuclein signals in the axon fibers of (B) nontreated and (C) PK-treated DL striatum sections of 18-month-old A53T/Aldh1a1+/+ and A53T/Aldh1a1−/− female mice (n = 3 animals per genotype). (D) Western blot shows the formation of α-synuclein-positive HMW bands in the striatum homogenate of 6-month-old A53T/Aldh1a1+/+ and A53T/Aldh1a1−/− mice. (E) The relative intensity of HMW α-synuclein bands (n = 3 female mice per genotype). (F) A Western blot of α-synuclein from the APBA pull-down experiment shows the enrichment of DOPAL-modified α-synuclein in 6-month-old A53T/Aldh1a1+/+ and A53T/Aldh1a1−/− mice. (G) The graph indicates an increase of accumulation of HMW DOPAL-modified α-synuclein in brains of A53T/Aldh1a1−/− mice compared with littermate A53T/Aldh1a1+/+ mice (n = 3 pairs females). *P < 0.05, ***P < 0.001.
rons were degenerated during aging. Since the expression of transgenic α-synuclein was comparable between these two populations of SNpc DA neurons, we conclude that a combination of Aldh1a1 deficiency, α-synuclein overexpression, and aging may contribute to the loss of ALDH1A1-negative neurons in these mice through a “multiple-hit” mechanism. Take collectively, these mouse studies suggest that a lack of Aldh1a1 is not sufficient to cause SNpc DA neuron loss in normal control and Aldh1a1–/– mice but that it contributes to the α-synuclein–induced SNpc DA neuron loss during aging. In line with this notion, genetic deletion of Aldhb1a1 exacerbates SNpc DA neuron loss in α-synuclein transgenic mice, whereas overexpression of ALDH1A1 is protective. Enhancement of ALDH1A1 activities may thereby serve as a potential therapeutic strategy for the treatment of SNpc DA neuron loss. It is worth pointing out that all mice used in this study were in C57/BL6 congenic background. Whether the same phenotypes would appear in different strains or species remain to be determined.

An early study demonstrates a potential pathogenic interaction between cytosolic dopamine and α-synuclein in causing cell death (17, 34). The autoxidation of cytosolic dopamine may produce cytotoxic quinones and free radicals. Particularly, quinone may covalently modify α-synuclein and favor the formation of more cytotoxic α-synuclein protofibrils (17). However, alterations of other enzymatic processes can also lead to the accumulation of cytotoxic aldehydes. In DA neurons, a battery of enzymes, including monoamine oxidases, ALDHs, and catechol-O-methyl transferases, are used to convert dopamine into the inactive metabolite, DOPAC. ALDH1A1, one of main ALDHs in the SNpc DA neurons, likely exerts its protective function through removal of the biogenic dopamine derivative DOPAL (12, 35). DOPAL is highly reactive and may modify and impair many cellular proteins critical for the normal function and survival of SNpc DA neurons (13). The presence of excessive α-synuclein in DA neurons may impair the synthesis, uptake, and degradation of dopamine (10, 36, 37); the increase of cytosolic dopamine may increase the likelihood of the formation of cytotoxic dopamine quinones and dopamine aldehydes, like DOPAL. DOPAL promotes α-synuclein polymerization in forming more cytotoxic α-synuclein aggregates (18, 21). Indeed, more α-synuclein aggregates were present in ALDH1A1-negative nigrostriatal DA neurons of α-synuclein transgenic mice. Additionally, genetic deletion of Aldh1a1 caused further accumulation of α-synuclein aggregates. Notably, the protective function of ALDH1A1 seems specific to α-synuclein–induced midbrain DA neuron loss. Aldh1a1+/+ and Aldh1a1–/– midbrain

Figure 7
Overexpression of ALDH1A1 protects midbrain DA neurons against α-synuclein–mediated cytotoxicity. (A) Representative images show cleaved caspase-3, TH, and human α-synuclein in control and A53T cultures on DIV8. Arrowheads indicate cleaved caspase-3–positive TH+ neurons. Arrows point to cleaved caspase-3–positive astrocytes. The death of astrocytes was caused by cytosine β-D-arabinofuranoside in the medium. (B) Expression of ALDH1A1 and TH in the A53T midbrain cultures infected with either AAV-ALDH1A1 or AAV-GFP virus. The noninfected or AAV-GFP virus–infected cultures serve as the control. Scale bar: 20 μm (A and B). (C) Survival rate (percentage) of TH-positive neurons treated with AAV-ALDH1A1 or AAV-GFP viruses. Six independent nTg and A53T cultures per condition were analyzed. (D) The number of surviving TH-positive neurons treated with vehicle DMSO, SIB1757, and DOX in control and A53T mouse midbrain neuron cultures. Seven independent nTg and A53T cultures per condition were analyzed. *P < 0.05, **P < 0.01, ***P < 0.001.
DA neurons showed similar vulnerability to MPP⁺-, glutamate-, and camptothecin-mediated cell death. Meanwhile, overexpression of ALDH1A1 did not rescue the α-synuclein–induced loss of cortical neurons. In line with the previous findings (17), our studies further support the notion that the reactive byproducts derived from the abnormal cytosolic dopamine oxidation, such as quinones and DOPAL, may cause the SNpc DA neuron loss by facilitating the formation of cytotoxic α-synuclein oligomers. It remains intriguing why ALDH1A1 is selectively expressed by a subpopulation of SNpc DA neurons. ALDH1A1 may not only oxidize reactive DOPAL, but also affect the steady level of dopamine in DA neurons (15, 16); a lack of ALDH1A1 in these neurons may slow down the catabolism of dopamine and enhance dopamine transmission. Future studies may be interesting to determine whether ALDH1A1-positive and -negative SNpc DA neurons have different connectivity and functionalities in regulating the physiological activities of neurons in the striatum for various motor controls. In addition, it is also intriguing why fewer ALDH1A1-positive DA neurons were found in the SNpc of 1-month-old A53T transgenic mice. An early study suggests that ALDH1A1 may be involved with the differentiation of midbrain DA neurons during development via producing retinoic acid (38). We speculate that the early onset of transgenic α-synuclein overexpression (10) may interfere with the expression and function of ALDH1A1, resulting in less production of ALDH1A1-positive DA neurons during development.

ALDH1A1-positive SNpc DA neurons were spared from degeneration in α-synuclein transgenic mice. On the contrary, in addition to degeneration of dorsal ALDH1A1-negative neurons, a more severe loss of VL ALDH1A1-positive SNpc DA neurons seems to be a prominent pathological feature of the postmortem PD brains (3). Notably, ALDH1A1-negative DA neurons seem to replace the positive ones in the ventral tier of SNpc in the mild PD cases, reflecting a decrease of ALDH1A1 expression in the ALDH1A1-positive DA neurons prior to the neuronal loss. A reduction of ALDH1A1 mRNA expression has also been reported previously in the SNpc of postmortem PD brains (22). However, the mechanism of reduced expression of ALDH1A1 mRNA in PD brains is unclear. It may reflect the loss of SNpc DA neurons, a reduction of ALDH1A1 mRNA expression in individual neurons, or a combination of both neuronal loss and gene expression decrease. In α-synuclein transgenic mice, both Aldh1a1 mRNA and protein levels were substantially decreased in DA neurons. However, ALDH1A1 remained detectable in the SNpc DA neurons in aged α-synuclein transgenic mice, which may explain why no significant loss of ALDH1A1-positive population of SNpc DA neurons was observed in these mice. Our findings suggest that ALDH1A1 itself is also a target in α-synuclein–mediated pathogenic processes in PD. The reduction of ALDH1A1 expression in PD may weaken the protective function of ALDH1A1 against DA neurodegeneration in the ventral tier of SNpc. In support of this notion, genetic ablation of Aldh1a1 exacerbates DA neuron loss in α-synuclein transgenic mice, whereas overexpression of ALDH1A1 is protective.

While ALDH1A1 plays an important role in protecting α-synuclein–induced loss of DA neurons, we cannot exclude the involvement of other genetic factors important for the survival of ventral ALDH1A1-positive DA neurons. A recent study suggests that calbindin-positive and -negative SNpc DA neurons show differential vulnerability to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment in mice (39). We carried out ALDH1A1, calbindin, and TH costaining in the midbrain sections of 2-month-old wild-type C57BL/6 mice (Supplemental Figure 1). There were a few calbindin-positive cells in the SNpc, which were distributed mainly in the medial area adjacent to the VTA. However, these calbindin-positive cells were negative to both TH and ALDH1A1 staining. The identity of these cells remains elusive. In the VTA, there were more calbindin-positive cells. However, the majority of these cells were not positive to either TH or ALDH1A1 staining. Our data do not support the notion that calbindin is a valid molecular marker for different subpopulations of SNpc DA neurons in mouse brains. It remains interesting to identify other genes selectively expressed by subpopulations of SNpc DA neurons, which may provide additional molecular insights into this rather unique pathophysiological property of ALDH1A1-positive DA neurons in PD-related neurodegeneration.

In summary, the present study identified ALDH1A1 as an important molecular marker for the subdivision of DA neurons in the SNpc that show differential susceptibility in PD-related DA neurodegeneration. Our findings also support a protective function of ALDH1A1 in maintaining the normal function and survival of SNpc DA neurons, proposing ALDH1A1 as a potential therapeutic target in preventing PD-related SNpc DA neuron loss.
Methods

Animals

Pitx3^{TRE2-CA} (Pitx3-tTA) knockin mice and tetO-A53T transgenic mice were created as described previously (10, 30). Aldh1a1^+/- (24) and CaMKII-tTA (40) mice were obtained from The Jackson Laboratory. The Pitx3^{TRE2-CA} mice were crossed with tetO-A53T transgenic mice to get Pitx3-tTA/tetO-A53T (A53T) double-transgenic mice. Pitx3^{TRE2-CA} and tetO-A53T transgenic mice were also crossed with Aldh1a1^+/- mice to generate Pitx3-tTA/Aldh1a1^+/- and tetO-A53T/Aldh1a1^+/- mice. These two lines of mice were intercrossed to get A53T/Aldh1a1^+/- and A53T/Aldh1a1^+/- animals. All of the mice were housed in a 12-hour-light/dark cycle and fed regular diet ad libitum. All mouse work follows the guidelines approved by the Institutional Animal Care and Use Committees of the National Institute of Child Health and Human Development, NIH.

Genotyping

Genomic DNA was prepared from tail biopsy using DirectPCR Lysis Reagent (Viagen Biotech Inc.) and subjected to PCR amplification using specific sets of PCR primers for each genotype, including Pitx3-tTA knockin mice (Pitx3-F: GACTGGCTTGGCCTGTCCCA and Pitx3-R: GTGCAGCGAGGCCCATGTC), tetO-A53T transgenic mice (PrpEx2-F: TACTGCTCATTTTGCCTGA and SNCA-R: TCCAGAAATTTCTCTCGTGG), Aldh1a1^+/- mice (Aldh1a1+/-m: CTATCGCCTTCTGCAAGTTCT and Aldh1a1+/-m: CTCCTGATCATTTAAGGGTCCACA), and Aldh1a1 wild-type mice (Aldh1a1+/-w: TAAAGACCTGATAAGGGCCTCA and Aldh1a1+/-w: ACGGTGCACAAAAATACATCTG).

Immunohistochemistry and light microscopy

As described previously (10), mice were sacrificed and then perfused via cardiac infusion with 4% paraformaldehyde in cold PBS. To obtain frozen sections, brain tissues were removed and submerged in 30% sucrose for 24 hours and sectioned at 30-μm thickness using a cryostat (Leica CM1950). Antibodies specific to TH (rabbit polyclonal, 1:1,000, Pel-Freez; mouse monoclonal, TH-2, 1:500, Sigma-Aldrich), α-synuclein (4D6, 1:500, Santa Cruz Biotechnology), and α-synuclein (syn211, 1:500, Santa Cruz Biotechnology), ALDH1A1 (1:500, Sigma-Aldrich), and calbindin (C9848, 1:500, Sigma-Aldrich) were used as suggested by manufacturers. Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibody (1:1,500, Invitrogen) was used to visualize the staining. Fluorescence images were captured using a laser scanning confocal microscope (LSM 510; Zeiss). The paired images in the figures were collected at the same gain and offset settings. After collection processing was applied uniformly to all paired images. The images were presented as either a single optic layer after acquisition in z-series stack or μ-scan at 0.8-μm intervals from individual fields or displayed as maximum intensity projections to represent confocal stacks.

PK digestion analysis

The frozen sections were pretreated with PK (50 μg/ml, Viagen Biotech Inc.) at 37°C for 30 minutes as described previously (19). Immunostaining and fluorescence images were performed as described above.

Image analysis

For the quantitative assessment of various marker protein accumulations and distributions, images were taken using identical settings and exported to ImageJ (NIH) for imaging analyses. Images were converted to an 8-bit color scale (fluorescence intensity from 0 to 255) using ImageJ. Areas of interest were first selected by Polygon or Freehand selection tools and then subjected to measurement by mean optical intensities. The mean intensity for the background area was subtracted from the selected area to determine the net mean intensity.

Stereology

According to the mouse brain in stereotaxic coordinates (41), a series of coronal sections across the midbrain (30 μm per section, every fourth section from bregma –2.54 mm to –4.24 mm) were chosen and processed for ALDH1A1 and TH staining, as described above, and visualized using a laser scanning confocal microscope (LSM 510; Zeiss). We examined 12–14 sections per brain. The images were captured as a projected layer at 20 μm (pinhole, 10 μm; interval, 10 μm, 2 layers) under ×10 magnification. The number of TH- and ALDH1A1-positive neurons was assessed using the Fractionation function of Stereo Investigator 10 (MicroBrightField Inc.). The sampling scheme was designed to have coefficient of error (CE) of less than 10% in order to get reliable results. A first count of samples was performed to achieve a total marking of >200 cells, which generally yields CE <10%. Once the initial cell count was completed, the CE was calculated. The counting parameters would be adjusted based on the CE value. To achieve CE <10%, normally 12 serial sections, with a total of 100 counting frames and, on average, 2 cells per frame would be counted. The final parameters for these studies were as follows: grid size, 200 × 200 μm; frame size, 50 × 50 μm. Three or more mice were used per genotype at each time point. Counters were blinded to the genotypes of the samples.

3D reconstruction of mouse midbrain DA neurons

According to the mouse brain in stereotaxic coordinates (41), a series of coronal sections across the midbrain (40 μm per section, every fourth section from bregma –2.54 mm to –4.24 mm, 10–12 sections per case) were processed for TH (1:1,000, Pel Freeze) staining overnight and subsequently with ABC reagents (Vector Laboratories) for an additional hour. Visualization was performed using a DAB Kit (SK-4100, Vector Laboratories) for 5 minutes at room temperature. The serial sections were used for the 3D reconstruction of midbrain using Stereo Investigator 3D reconstruction software (MicroBrightField Inc.). Reconstruction was analyzed for stereology using the Neurolucida Explorer, which returned the nearest neighbor for the distance of DA neurons and the enclosed volume of SNpc.

Human brain tissues

Rapidly autopsied SNpc sections (within 3 hours of the postmortem interval) were obtained from the Brain and Body Donation Program of the Banner Sun Health Research Institute (42) as well as the brain bank of Johns Hopkins University School of Medicine (Supplemental Table 1). Subjects or their legal representatives provided informed consent, and studies were approved by the Banner Health and Johns Hopkins Institutional Review Boards. The diagnosis of PD was made based on clinicopathological criteria, including characteristic clinical features, and on the presence of Lewy bodies within pigmented neurons lost in the substantia nigra. Subjects with PD were divided into two groups on the basis of the presence or absence of clinically documented dementia. Nonpathological controls were selected based on the absence of cognitive impairment, Parkinsonism, and Lewy bodies. Tissues of patients with autosomal recessive juvenile Parkinsonism, a relatively rare syndrome that shares many features of Parkinsonism without the presence of Lewy bodies or Lewy neurites, were excluded from this study.

Human SNpc immunohistochemistry and 3D reconstruction

Human midbrain sections were from the John Hopkins University Brain Resource Center and Banner Sun Health Research Institute and are described in Supplemental Table 1. According to the Atlas of the Human Brain (43), transverse SNpc sections (50 μm per section, every 20th section from bregma 16.0 mm to 27.8 mm; 10 sections per case) from 9 control subjects and 10 patients with PD (between 71 and 90 years old) were deep-arafinized and rehydrated in ethanol series. Antigen retrieval techniques
were then used (sections were microwaved at full power in TBS buffer [pH 8.8] for 15 minutes twice) before H2O2 treatment. Sections were incubated with rabbit anti-ALDH1A1 polyclonal antibody (1:500, Millipore) overnight and subsequently with the ABC reagents (Vector Laboratories) for an additional hour. Visualization was performed using a DAB Kit (SK-4100, Vector Laboratories) with nickel enhancement for 5 minutes at room temperature. After DAB, all sections were dehydrated and sealed with coverslips.

The 3D reconstruction of human SNpc was performed using Stereo investigatory (MicroBrightField Inc.). Nigrostriatal DA neurons were marked by the presence of NM in the soma. The ALDH1A1+/NM+ and ALDH1A1−/NM− DA neurons were designated as 2 different markers. Contours of dorsal, VL, VM, and PL parts were created according to the literature (3) and the supernatants were loaded on the 4% to 12% NuPAGE Bis-Tris gel electrophoresis (Invitrogen) using MES running buffer. After transfer to nitrocellulose membranes, the membranes were immunoblotted with the appropriate dilutions of the primary antibodies α-synuclein (C20, 1:1,000; Sigma-Aldrich), and DARPP32 (Cell Signaling Technology) at 4°C. Signals were visualized by enhanced chemiluminescence development and quantified with ImageJ.

Behavior tests

Open-field test. As described previously (45), the ambulatory and rearing activities of mice were measured by the Flex-Field activity system (San Diego Instruments). Flex-Field software was used to trace and quantify mouse movement in the unit as the number of beam breaks per 30 minutes.

Rotarod test. As described previously (45), mice were placed onto a rotating rod with autoacceleration from 0 to 40 rpm for 5 minutes (San Diego Instruments). The length of time the mouse stayed on the rotating rod was recorded across 10 trials.

Generation of AAV-overexpressing ALDH1A1

Human ALDH1A1 full-length cDNA (Addgene plasmid 11610) was subcloned into pAAV-NMCS vector (46), which was provided by Joanna Janowsky from College of Medicine, Houston, Texas, USA. The Gene Core Laboratory at Baylor College of Medicine prepared the AAV-ALDH1A1 and AAV-GFP viruses for the infection experiments.

Midbrain neuronal culture; AAV viral infection; MPP+, glutamate, camptothecin treatment; and immunocytochemistry

Midbrain neuron-enriched cultures were prepared from postnatal day 0 pups from tetO-AS3T and Pitx3-tTA crossbreeding (10). The pups were under the DOX treatment to inhibit the expression of transgenic α-synuclein during embryonic stages. Midbrain tissues containing SNpc and VTA, without the meninges and blood vessels, were subjected to papain (5 U/ml, Worthington) digestion for 40 minutes at 37°C. The digested tissue was carefully triturated into single cells using increasingly smaller pipette tips. Cells were centrifuged at 250 g for 5 minutes and resuspended in warm Basal Medium Eagle (Sigma-Aldrich) supplemented with 5% heat-inactivated fetal bovine serum, 1× B27, 1× N2, 1× GlutaMAX (Invitrogen), 0.45% D-glucose (Sigma-Aldrich), 10 U/ml penicillin, and 10 μg/ml streptomycin (Invitrogen). Dissociated cells from each midbrain were equally divided (~2.5 × 10^5 cells) were plated on each coverslip and plated onto five 12-mm round coverslips precoated with poly-D-lysine and laminin (BD Bioscience), and maintained at 37°C in a 95% O2- and 5% CO2-humidified incubator. 24 hours after seeding, the cultures were switched to serum-free medium supplemented with 5 μM cytosine β-D-arabinofuranoside (Sigma-Aldrich), which was used to suppress the proliferation of glia. Cells in one sister coverslip were maintained in the presence of 2.5 mg/ml DOX after plating. For AAV viral infection, after 3 days in vitro (DIV3), cells without DOX treatment were infected with AAV-expressing GFP and ALDH1A1 at the multiplicity of infection of 10^7. AAV-infected cells were then labeled with primary antibodies against TH (Santa Cruz Biotechnology, 1:500) and/or ALDH1A1 (Sigma-Aldrich, 1:1,000) overnight at 4°C in a humidified chamber.

For ALDH1A1 activator SIB1757 (Sigma-Aldrich) treatment, after DIV5, cells without DOX treatment were treated with DMSO or 10 μM SIB1757. On DIV8, cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS for 15 minutes, permeabilized by 0.1% Triton X-100 for 5 minutes, and blocked in 10% nonimmune donkey serum (Invitrogen) for 1 hour at room temperature. SIB1757-treated cells were stained with antibody against...
cleaved caspase-3 (Cell Signaling Technology, 1:100), TH (Abcam, 1: 200), and human α-synuclein (Santa Cruz Biotechnology, 1:500). After 3 washes with PBS, donkey-derived secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 546, and/or Alexa Fluor 647 (Invitrogen; 1:1,000) were applied and incubated for 1 hour at room temperature in the dark. After extensive washes, coverslips were mounted on glass slides with Prolong Gold antifade reagent containing DAPI (Invitrogen), and fluorescence signals were detected using a laser scanning confocal microscope (LSM 510; Zeiss). The total number of TH-positive neurons on each sister coverslip was counted under a ×25 objective.

For MPP+, glutamate, and camptothecin treatment, midbrain neurons derived from Aldh1a1-/- and Aldh1a1+/+ P0 pups were treated with these cell death inducers on DIV12 at dosages ranging from 0 to 1,000 μM. After 24 or 48 hours of treatment, neurons were fixed with 4% PFA and immunostained with TH antibody and secondary antibody. The total number of all TH-positive neurons on each of the sister coverslips was counted under a ×25 objective.

The survival rate of TH-positive neurons was calculated by dividing the number of TH-positive neurons on each coverslip by the number of TH-positive neurons on the corresponding NGFP coverslip (with an average number of TH+ neurons per coverslip around 225) infected with AAV-GFP (for viral infection) or treated with vehicle (for SIB1757, MPP+, glutamate, and camptothecin treatment).

**Cortical neuronal culture, AAV viral injection, and MTT assay**

Primary cortical neurons were cultured from P0 CaMKII-tTA/tetO-AS3T pups (30). 1 x 10⁴ neurons were seeded per well in 96-well plate for MTT assay or 1.5 x 10⁵ neurons were seeded per well in 6-well plate for Western blot analysis. Neurons were either treated with 2.5 μg/ml DOX to inhibit the expression of transgenic α-synuclein from DIV0 or infected with 1 x 10⁷ AAV8-GFP or AAV8-ALDH1A1 on DIV1. On DIV7, the neuron loss in the 96-well plate was measured by MTT assay (Sigma-Aldrich). AS3T overexpression level was confirmed by Western blot in a 6-well plate with or without DOX treatment (Supplemental Figure 5).

**LCM and quantitative reverse transcriptase–PCR assay**

Brains of Ptx3-ATA/tetO-H2B-GFP control double-transgenic and Ptx3-ATA/tetO-H2B-GFP/tetO-AS3T triple-transgenic mice (10) were quickly dissected out, and the frozen brains were sectioned at 20-μm thickness by a cryostat onto a PAN membrane frame slide (Applied Biosystems) and stored at –80°C until LCM. The GFP-positive cells in the SNpc were identified by a cryostat onto a PAN membrane frame slide (Applied Biosystems) separately at the following working parameters: spot size, 7–25 μm; power, 50–70 mW; duration, 20–40 μs. The total RNA was extracted with the PicoPure Isolation Kit (Applied Biosystems) using the protocol provided by the manufacturer. The cDNA was synthesized from 50 ng RNA by the First-Strand Kit (QIAGEN) after genomic DNA elimination. The SYBR green real-time PCR detection method was used to quantitate the ALDH1A1 expression levels in the control and AS3T transgenic SNpc DA neurons, which were normalized by β-actin (Actb) expression. The Aldh1a1 and Actb primers used were from QIAGEN and tested by the manufacturer.

**Statistics**

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.). Data are presented as mean ± SEM. Statistical significance was determined by comparing means of different groups and conditions using unpaired 2-tailed Student’s t test, 1-way ANOVA with post-hoc Tukey test, and 2-way ANOVA with post-hoc Bonferroni test.

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