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β2-Adrenoreceptor is a Regulator of the α-Synuclein Gene Driving Risk of Parkinson's Disease

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Supplementary Materials

Materials and Methods
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Abstract

Copy number mutations implicate excess production of α-synuclein as a possibly causative factor in Parkinson's disease (PD). Using an unbiased screen targeting endogenous gene expression, we discovered that the β2-adrenoreceptor (β2AR) is a regulator of the α-synuclein gene (*SNCA*). β2AR ligands modulate *SNCA* transcription through histone 3 lysine 27 acetylation of its promoter and enhancers. Over 11 years of follow-up in 4 million Norwegians, the β2AR agonist salbutamol, a brain-penetrant asthma medication, was associated with reduced risk of developing PD (rate ratio, 0.66; 95% confidence interval, 0.58 to 0.76). Conversely, a β2AR antagonist correlated with increased risk. β2AR activation protected model mice and patient-derived cells. Thus, β2AR is linked to transcription of α-synuclein and risk of PD in a ligand-specific fashion and constitutes a potential target for therapies.

The brains of most patients with Parkinson's disease (PD) are riddled with intracellular accumulations of α-synuclein protein known as Lewy bodies. Triplication or duplication of the wild-type α-synuclein gene (*SNCA*) locus is sufficient to cause familial PD (1, 2). In these patients, copies of functionally normal *SNCA* mRNA and α-synuclein protein are increased by about 50 to 100% (2, 3). Even smaller increases in α-synuclein transcription may play an analogous role in patients with sporadic disease carrying potential regulatory variants in this gene (4).

Traditionally, drug development in PD has focused on clearance of α-synuclein protein, blockade of its transformation into toxic species, or amelioration of its downstream consequences. In contrast, we hypothesized that chemical compounds designed to reduce the transcription of the *SNCA* gene could make it possible to prevent or slow down the disease process in selected patients, but this idea lacked a druggable target. Regulation of *SNCA* expression appears to include GATA transcription factor occupancy of evolutionarily conserved enhancers in intronic regions of *SNCA* (5) and, possibly, the NGF (nerve growth factor) and bFGF (basic fibroblast growth factor) pathways (6), methylation (7), and micro RNAs (8). However, none of these candidates can be easily targeted by available medicines.

Drug screen targeting endogenous *SNCA* expression identifies β2AR agonists

We developed a high-throughput gene expression assay for endogenous human *SNCA* expression in situ in neuronal cells. This is an alternative approach to construct-based reporter assays, which typically do not fully represent the integrated microcircuit of promoters, enhancers, and histone marks that naturally regulate gene expression in a human cell. Human SK-N-MC neuroblastoma cells were cultured and drug-treated in 384-well plates, and relative endogenous *SNCA* mRNA expression was assayed.

SNCA expression–lowering compounds were identified in a four-stage study design (Fig. 1) consisting of screening, replication, and confirmation of transcript expression, followed by an enzyme-linked immunosorbent assay (ELISA) stage for quantification of protein

expression. We screened 1126 compounds, including drugs approved by the U.S. Food and Drug Administration (FDA) and a diverse set of natural products, vitamins, health supplements, and alkaloids (data S1 and fig. S1). SK-N-MC cells were treated with each compound for 48 hours. Forty-one compounds were included in the replication stage: Thirty-five compounds, including the selective β2-adrenoreceptor (β2AR) agonist metaproterenol, lowered *SNCA* expression by more than 35% in the screening stage; six related drugs, including the selective β2AR agonists clenbuterol and salbutamol, were added at the replication stage ("hit expansion"). Four compounds had P values ≤ 0.005 (two-tailed Student's t test) in the confirmation stage and also lowered α-synuclein protein abundance (determined by ELISA) in SK-N-MC cells ($P \leq 0.05$; two-tailed Student's t test, comparing with vehicle) (Fig. 1A). Unexpectedly, three of these hits were β2AR agonists (Fig. 1B), and these were prioritized for further investigation.

Treatment with metaproterenol reduced *SNCA* mRNA abundance in SK-N-MC cells compared with that in control cells ($P = 0.005$; two-tailed Student's t test) in the confirmation stage (fig. S2A) and was further verified (fig. S2B). Treatment with clenbuterol (fig. S2C) and salbutamol (fig. S2D) also had similar effects on relative *SNCA* mRNA abundance. Thus, we concluded that β2AR activation may regulate endogenous *SNCA* expression in SK-N-MC cells. Interestingly, the screen highlighted riluzole hydrochloride (fig. S1E) as a fourth hit. This compound is FDA-approved for modification of amyotrophic lateral sclerosis and has been shown to attenuate dopaminergic neurodegeneration in a 6-hydroxydopamine rat model of PD (9).

β2AR activation selectively modulated the expression of *SNCA* without adversely affecting neuronal cell viability or housekeeping gene expression (fig. S3) (10). As expected, the effects of β2AR agonists on *SNCA* expression were dependent on cellular context (fig. S4). For example, in human erythroleukemia cells, which express *SNCA* mRNA but lack β2AR (fig. S4A), and in neuronal SH-SY5Y cells, which transcribe β2AR but express low levels of *SNCA* mRNA (fig. S4B), agonists did not influence *SNCA* expression (fig. S4, C and D). These results are consistent with the specificity of our observations.

We used a sensitive ELISA and antibodies against α-synuclein (11) to determine whether the modulation of *SNCA* mRNA expression by β2AR translates into changes in α-synuclein protein abundance. In rat primary cortical neurons, endogenous *SNCA* mRNA (Fig. 1C) and α-synuclein protein (Fig. 1D) levels were significantly, but modestly, reduced in response to β2AR activation by metaproterenol ($P < 0.005$ and 0.05, respectively), clenbuterol ($P < 0.005$), or salbutamol ($P < 0.005$), compared with controls [analysis of variance (ANOVA) with Tukey's].

β2AR agonists lowered *SNCA* expression in a dose- and time-dependent manner (10) (fig. S5). Increasing concentrations of clenbuterol (5, 10, and 20 μM) correlated with a decrease in *SNCA* mRNA (Fig. 1E) and α-synuclein protein (Fig. 1F) levels in SK-N-MC cells. Similarly, metaproterenol and salbutamol lowered *SNCA* mRNA expression in a dose-dependent manner ($P < 0.005$; ANOVA with Tukey's) (fig. S6).

β2AR activation reduces *Snca* expression in mouse substantia nigra

PD preferentially affects dopaminergic neurons in the substantia nigra. We examined the effects of the selective β2AR agonist clenbuterol (which can be efficiently administered intraperitoneally) to probe the effects of β2AR activation on *Snca* expression in the substantia nigra of wild-type C57BL/6J mice. As expected (12, 13), clenbuterol crossed the blood-brain barrier, and its brain/plasma ratio increased with doses of 1, 5, or 10 mg of drug per kilogram of body weight (Fig. 2A).

Intraperitoneal injection of 10 mg/kg, administered for 24 hours, resulted in the highest brain/plasma ratio (Fig. 2A) and brain concentration (Fig. 2B) and induced a significant reduction in nigral α-synuclein protein and mRNA levels ($P < 0.05$; two-tailed Student's t test) (Fig. 2C). We then performed a larger, randomized, placebo-controlled trial in mice to determine whether clenbuterol is efficacious in lowering α-synuclein expression in the substantia nigra of wild-type mice. Mice were euthanized after 24 hours of acute drug treatment. β2AR activation lowered the expression of endogenous α-synuclein protein and mRNA levels in the PD vulnerable substantia nigra ($P = 0.01$; two-tailed Student's t test) (Fig. 2D). This was confirmed by Western blotting with various antibodies against α-synuclein (fig. S7). Overall, β2AR agonist treatment reduced *SNCA* expression in rodent neurons and substantia nigra.

Bidirectional modulation of *SNCA* expression by β2AR

We examined *Snca* expression levels in primary neurons derived from mice carrying a deletion of the β2AR gene (*Adrb2*). Endogenous *Snca* mRNA and α-synuclein protein levels were increased by 100 and 120%, respectively, compared with those in controls ($P = 0.004$ and 0.01, respectively; Student's t test) (Fig. 2, E and F). In accord, silencing of β2AR in human SK-N-MC cells increased *SNCA* mRNA and α-synuclein protein levels (Fig. 2, G and H).

Moreover, chemical antagonism of β2AR with propranolol, a well-characterized b-blocker, in SK-N-MC cells similarly increased endogenous *SNCA* mRNA and α-synuclein protein levels ($P = 0.00001$ and 0.001, respectively; two-tailed Student's t test) (Fig. 2, I and J, and fig. S8). Conversely, transient transfection of SK-N-MC cells with *ADRB2* constructs reduced endogenous *SNCA* mRNA levels relative to those of controls ($P = 0.01$) (Fig. 2K). Genetic silencing of β2AR or cotreatment with propranolol blocked clenbuterol's *SNCA* expression–lowering effects (Fig. 2, L to O). Collectively, these internally consistent data suggest that β2AR modulation is sufficient for altering endogenous *SNCA* expression and necessary for mediating the effects of β2AR ligands on endogenous *SNCA* expression.

β2AR regulates transcription of human *SNCA* through H3K27 acetylation

SNCA transcription appears to be finely regulated through a classical promoter spanning the non– protein-coding exon 1 and intron 1 at the 5′ end of the *SNCA* locus and through enhancers in the long intron 4 (Fig. 3A) (5). We clarified the endogenous *SNCA* promoter and putative enhancer sites by CAGE (cap analysis gene expression) in human PD relevant substantia nigra and by integrative genomics (Fig. 3A) (10). Histone 3 lysine 27 acetylation

(H3K27ac) signals (indicative of active enhancer elements) were observed at the promoter and enhancer regions (Fig. 3A). Because β2AR stimulation has been implicated in regulating *WNK4* transcription through histone acetylation in renal cells (14), we hypothesized that β2AR activation may regulate *SNCA* transcription through an analogous mechanism.

Clenbuterol treatment reduced H3K27ac across the promoter (site 1, Fig. 3A) and two putative intronic enhancers (sites 2 and 3, Fig. 3A), compared with vehicle treatment ($P < 0.05$; one-way ANOVA with Tukey's). Conversely, the β-blocker propranolol increased H3K27ac across these putative regulatory sites (Fig. 3A) ($P < 0.05$). Consistently, the known histone deacetylase inhibitor valproic acid (15) increased H3K27ac (Fig. 3A). Western blotting with an antibody against H3K27ac confirmed our hypothesis (Fig. 3B). Clenbuterol treatment resulted in a correlated decrease in H3K27ac levels and relative *SNCA* mRNA abundance (Fig. 3B). Conversely, treatment with valproic acid resulted in an increase in H3K27ac levels and relative *SNCA* mRNA abundance, compared with vehicle treatment (Fig. 3B). Inhibition of H3K27 deacetylation (by cotreatment with valproic acid) abrogated the β2AR agonist effect on *SNCA* expression (Fig. 3C). Thus, β2AR regulates the transcription of α-synuclein in correlation with H3K27ac across the promoter and enhancers in the human *SNCA* locus.

β2AR ligands are associated with risk of PD in Norwegians

We evaluated the effects of β2AR activation in two nationwide, longitudinal analyses of incident PD in Norway; a mouse model of MPTP (N-methyl- 4-phenyl-1, 2, 3, 6-tetrahydropyridine)–induced human parkinsonism; and an iPSC (induced pluripotent stem cell)–derived neuronal culture system from a patient with autosomal dominant PD due to a triplication of the *SNCA* locus. The Norwegian Prescription Database (NorPD) contains complete information on all prescribed drugs dispensed at pharmacies to individuals in Norway since 2004 (16). Given that β2ARmodulates *SNCA* expression, we hypothesized that use of β2AR ligands would affect PD risk. We thus tested salbutamol and propranolol, respectively the most commonly used β2AR agonist and antagonist in Norway, as time-dependent covariates in two separate Cox proportional hazard models. We adjusted for sex, age, and level of education and included the total Norwegian population alive on 1 January 2004 as the study population (n = 4.6 million). We observed a yearly incidence rate of PD similar to that found in a recent clinical incidence study in Norway (10, 17). Salbutamol was associated with decreased risk of PD, with a rate ratio of 0.66 [95% confidence interval (CI), 0.58 to 0.76] (Tables 1 and 2, Fig. 4A, and fig. S9). Propranolol was associated with a markedly increased risk of PD, with a rate ratio of 2.20 (95% CI, 1.62 to 3.00) (Table 1 and Fig. 4B).

The most common indication for salbutamol in our database was asthma. Smoking has been associated with decreased risk of PD (18). Tobacco exposure is also associated with early childhood asthma (19). If smoking explained the reduced risk associated with salbutamol, we would expect to see a similarly reduced risk for other asthma drugs not acting on β2AR. However, inhaled corticosteroids, which are frequently prescribed for asthma, did not reduce the PD risk (rate ratio, 0.95; 95% CI, 0.80 to 1.12) (table S1) after adjusting for salbutamol

use and level of education. Further, adjusting for education, which is strongly associated with smoking habits in Norway (20), we observed only a slight change in the effect of salbutamol (Table 1). Thus, it is unlikely that smoking can fully explain the association between salbutamol and PD.

Propranolol is used to treat cardiovascular diseases and essential tremor, which might be misdiagnosed as a first sign of PD. To reduce this source of bias, we excluded all individuals with an indication of essential tremor or other neurological diseases and included only those with cardiovascular diagnoses. Moreover, we introduced a time lag between time of first exposure to propranolol and PD onset. Using time lags of 1 and 2 years only slightly reduced the effect estimates (rate ratio reduced from 2.20 to 1.82). This makes it unlikely that reverse causality explains a major part of this association.

β2AR activation protects MPTP model mice

In addition to α-synuclein, chemicals such as MPTP (21, 22) and rotenone (23, 24) are implicated in the mechanism of sporadic PD. These chemicals inhibit the flow of electrons through complex I of the electron transport chain and foster buildup of superoxide and other reactive oxygen species, particularly in dopamine neurons (22, 25, 26). We tested whether clenbuterol treatment could protect against MPTP-induced degeneration of tyrosine hydroxylase–positive (TH+) neurons in the substantia nigra pars compacta (SNpc) of a mouse model of PD (10, 22). Clenbuterol treatment abrogated the MPTP-induced loss of TH + neurons (Fig. 4, C and D) and, importantly, also blocked the loss of cresyl violet–stained cells in the SNpc (Fig. 4E and fig. S10).

β2AR agonist in patient-derived cells carrying a *SNCA* triplication

Triplication of the *SNCA* locus causes autosomal dominant PD (1, 2), with iPSC-derived neurons constitutively overexpressing endogenous α-synuclein (27). Increased levels of wild-type α-synuclein cause mitochondrial impairment and an increase in superoxide and other reactive oxygen species (28, 29), possibly because of interference with mitochondrial protein import (30). We tested whether clenbuterol may be helpful in normalizing *SNCA* expression levels in human iPSC derived neuronal cells of a patient carrying the *SNCA* triplication. *SNCA*-triplication iPSC-derived neuronal precursor cells were treated with clenbuterol (20 μM), and endogenous *SNCA* mRNA expression and α-synuclein protein levels were significantly reduced ($P < 0.005$ and 0.05, respectively; two-tailed Student's t test) (Fig. 4F). Similarly, *SNCA* expression was reduced in *SNCA*-triplication iPSC-derived neurons cultured for 8 weeks and then treated with clenbuterol (20 μM) for 3 days (fig. S11).

Furthermore, PD patient–derived neuronal precursor cells carrying the pathogenic *SNCA* locus triplication show increased mitochondria associated superoxide production and reduced viability under exposure to the environmental mitochondrial complex I toxin rotenone (28). Clenbuterol treatment ameliorated this increased mitochondria-associated superoxide production (Fig. 4G) and increased viability (Fig. 4H), similarly to partial *SNCA* knockdown (28).

Discussion

We found effects of β2AR activation in two epidemiologic analyses, in mice modeling neurotoxin induced human parkinsonism, and in iPSC-derived neuronal cultures modeling *SNCA* dosage and rotenone toxicity. We propose a model in which β2AR antagonists increase *SNCA* expression through H3K27 acetylation, resulting in α-synuclein accumulation, mitochondrial oxidative stress, dopaminergic neurodegeneration, and increased risk of PD. In contrast, we expect β2AR agonists to promote dopamine neuron health by reducing *SNCA* expression (through H3K27 deacetylation) and mitochondrial free radicals. This may benefit nigral dopamine neurons, which are prone to mitochondrial bioenergetics dysfunction even at early stages of Lewy body neuropathology (31) and are preferentially vulnerable to mitochondrial complex I toxins (22). There is precedent for β2AR stimulation acting as a regulator of transcription (14). β2ARs are expressed in the substantia nigra and cortex (32), regions that are progressively affected in PD. The ligand-specific regulatory mechanism that we uncovered is consistent with the clinical association in Norway, where the selective β2AR agonist salbutamol (typically prescribed for asthma) was associated with a reduced risk of PD, whereas the β2AR antagonist propranolol (commonly used for hypertension) was associated with increased risk.

We demonstrate associations of β2AR with neuronal *SNCA* expression and risk of PD. It is important to note that association does not imply causation. β2AR agonists are not currently FDA-approved for PD treatment. Cardiovascular disease can be exacerbated by β2AR agonists. Evaluation in additional populations and in clinical trials will be required to determine whether the insights gained in this work can be translated to patients with PD. The described regulatory pathway and the impacts of various compounds present a new view of *SNCA* biology and offer clues for medicinal chemistry and drug repurposing. Our screen targeted neuronal *SNCA*; however, β2AR may have additional beneficial effects on glia and inflammation (12, 33). A complete chart of the pathway components linking β2AR to PD pathobiology can now be realized and might inspire more potent and PD-specific interventions.

Our study presents a path to drug development that is distinct from traditional approaches. Targeting the endogenous expression of a human disease gene may be a useful strategy for other diseases attributed to copy number variation or regulatory variants. The drug development pipeline tested in this study could be more generally applicable to rapid discovery and translation of therapeutics for other brain diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Foundation (to C.R.S.), Prinses Beatrix Spier Fonds (to P.H.), the American Parkinson's Disease Association (to T.B.), the Parkinson's Disease Foundation (to T.B.), the Branfman Family Foundation (to J.C.R.), the Canadian Institute of Health Research (to D.S.P.), Brain Canada/Krembil Foundation (to D.S.P.), the Heart and Stroke Foundation of Canada (to D.S.P.), the Multiple System Atrophy Coalition (to V.K.), and Harvard NeuroDiscovery Center (to V.K.). B.W.H. has applied for a related U.S. patent. C.R.S. is named as inventor on patent application 62487541 submitted by Brigham and Women's Hospital that relates to modifications and combinations of b-adrenoreceptor agonists as potential therapeutics for Parkinson's disease. NorPD data are accessible by application at http://norpd.no.

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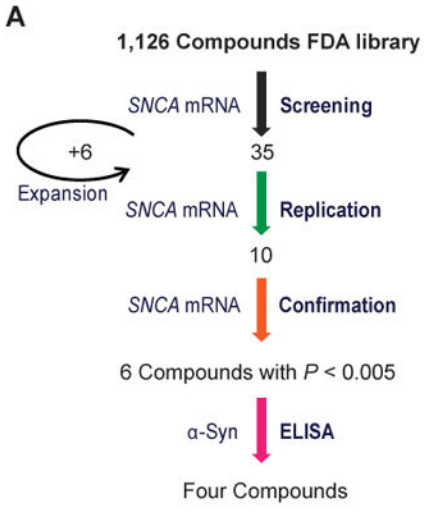
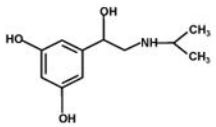
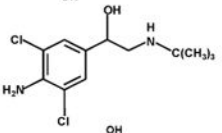
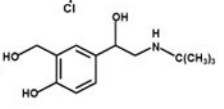


B

No.	Name	Class	Structure	FDA approved	Indication	Blood-brain penetrant
1.	Metaproterenol	β2–Adrenoreceptor Agonist		Yes	Asthma	No
2.	Clenbuterol	β2–Adrenoreceptor Agonist		No	Asthma	Yes
3.	Salbutamol	β2–Adrenoreceptor Agonist		Yes	Asthma	Yes

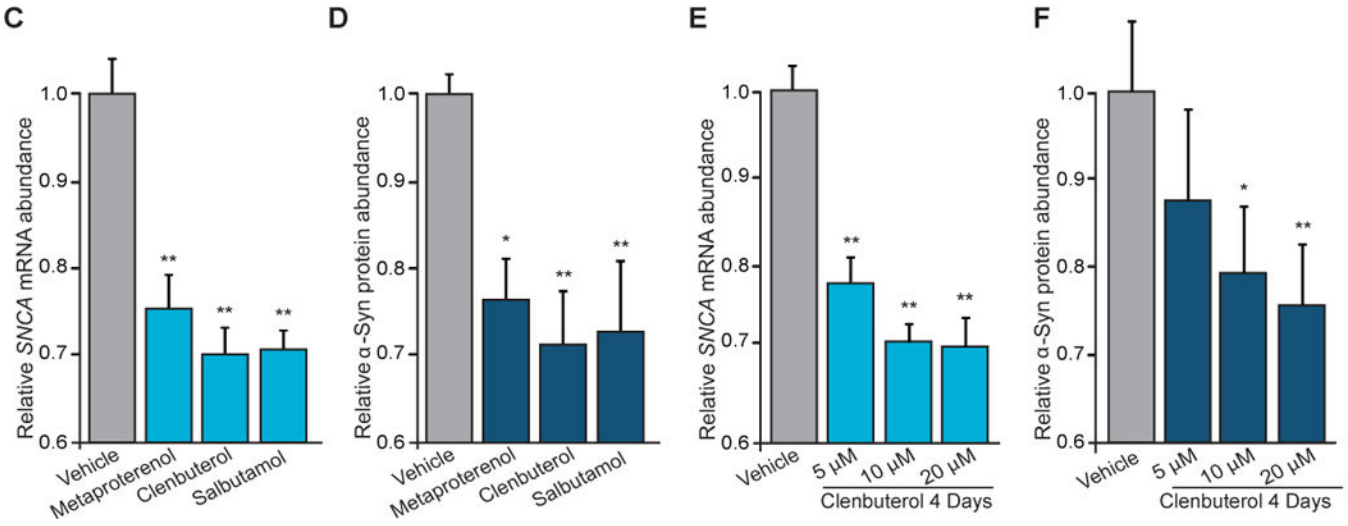


Fig. 1.

A screen of endogenous neuronal gene expression reveals β2AR as a regulator of *SNCA*. (A) Four out of a total of 1126 FDA-approved drugs and other compounds lowered the relative abundance of endogenous *SNCA* mRNA and α-synuclein protein (α-Syn) in SK-N-MC cells. (B) These included three selective β2AR compounds, whose chemical and clinical characteristics are shown. (C and D) The β2AR agonists metaproterenol (5 μM), clenbuterol (20 μM), and salbutamol (10 μM) also reduced the relative abundance of endogenous *SNCA* mRNA (C) and α-Syn protein (D) in rat primary cortical neurons (n = 4). (E and F) β2AR agonists lowered the expression of *SNCA* mRNA (E) and α-Syn protein (F) in a dose-dependent manner in neuroblastoma cells (n = 6 to 8). Means ± SEM are shown. * $P < 0.05$; ** $P < 0.005$; one-way ANOVA with Tukey's.

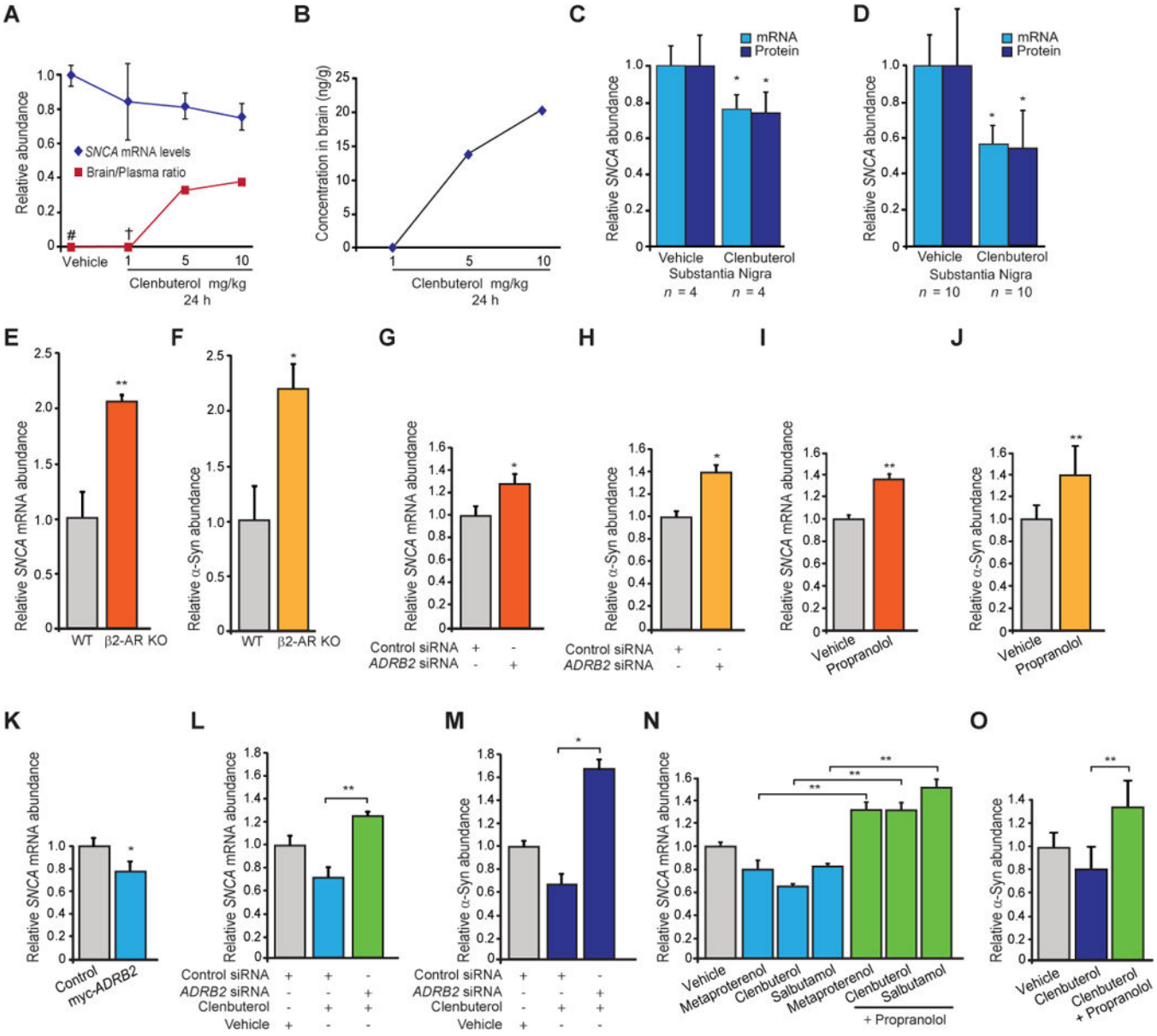


Fig. 2.

Bidirectional regulation of endogenous *SNCA* expression by β2AR modulation in vivo and in vitro. (A) Clenbuterol brain/plasma ratio in mice (red) and corresponding *SNCA* mRNA levels in the PD-vulnerable substantia nigra (blue). #Drug concentration below the quantifiable limit in brain and plasma. †Drug concentration below the quantifiable limit in brain. (B) Clenbuterol concentration in mouse brains. (C and D) β2AR activation lowered the expression of endogenous *SNCA* in the substantia nigra of mice in the dose-finding (C) and controlled (D) trials for 24 hours. (E to J) Knockout of the β2AR gene (Adrb2) in mouse primary neurons [(E) and (F); n = 6 to 9], silencing of β2ARs with RNA interference in human SK-N-MC cells [(G) and (H); n = 3], or chemical inhibition of β2ARs by the β-blocker propranolol in SK-N-MC cells [(I) and (J); n = 8 to 12] consistently increased the expression of *SNCA* mRNA [orange bars in (E), (G), and (I)] and α-Syn protein [yellow

bars in (F), (H), and (J)]. (K) Transient transfection of SK-N-MC cells with *ADRB2* constructs resulted in a reduction in endogenous *SNCA* mRNA levels, compared with those in cells transfected with empty vector (n = 6). (L to O) β2AR is necessary for mediating the effects of β2AR ligands on endogenous *SNCA* expression. Silencing of the β2AR gene abrogated the clenbuterol-induced reduction in *SNCA* mRNA and α-Syn protein expression [(L) and (M); n = 3]. Cotreatment with the β2AR antagonist propranolol abrogated the *SNCA* mRNA–lowering effects of metaproterenol, clenbuterol, and salbutamol [(N); n = 5 to 6]. Cotreatment with propranolol also abrogated the β2AR agonist–induced change in α-Syn protein levels [(O); n = 8 to 12]. siRNA, small interfering RNA. Means ± SEM. * $P < 0.05$; ** $P < 0.005$; two-tailed Student's t test [(C) to (K)] or one-way ANOVA with Tukey's [(L) to (O)].

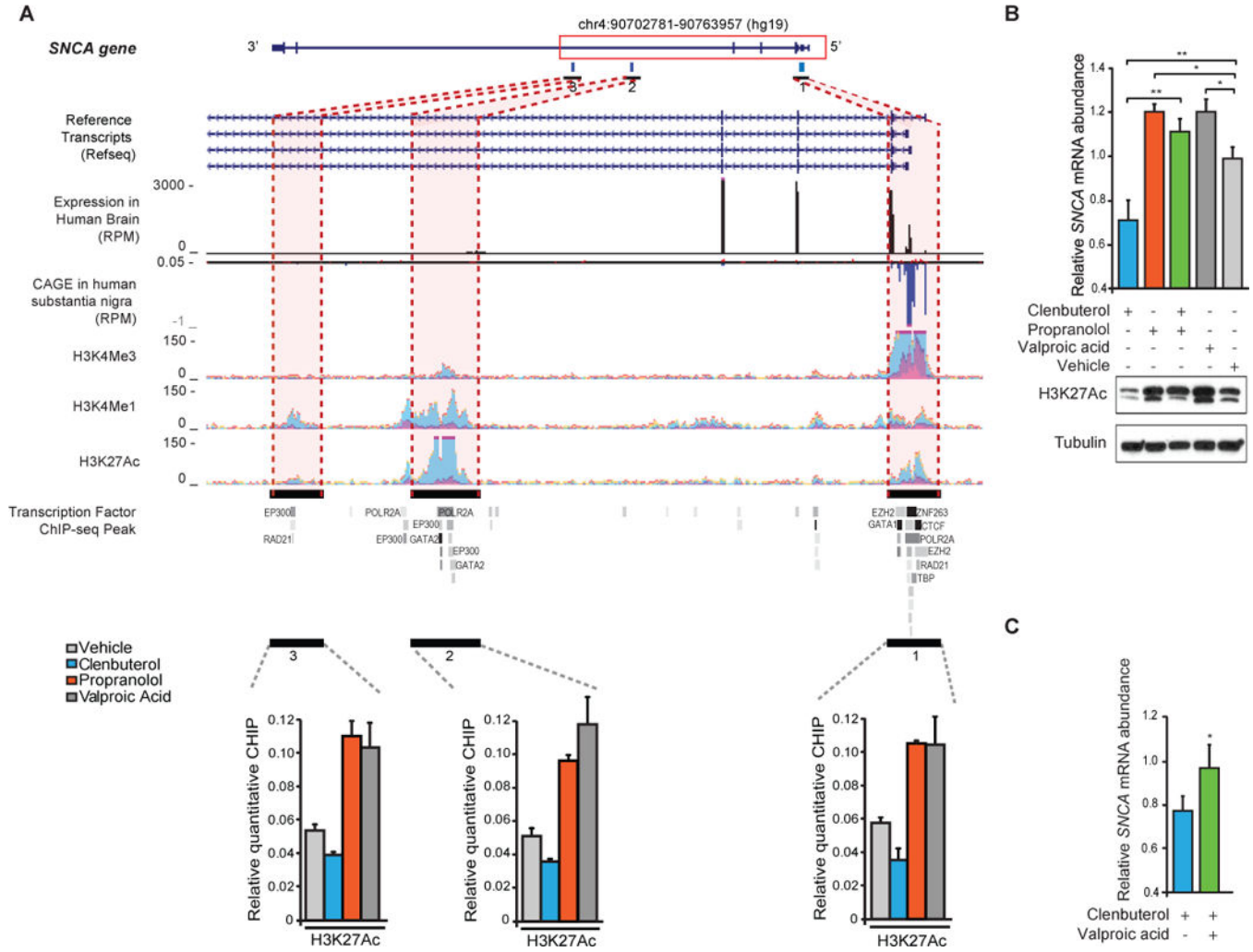


Fig. 3.

β2AR regulates the transcription of *SNCA* through H3K27 acetylation (H3K27ac) across the *SNCA* promoter and two enhancers in intron 4. (A) The *SNCA* gene, tracks for RefSeq transcripts, normalized read density of RNA sequencing in the human brain (34), CAGE in human substantia nigra (10), histone modifications (H3K4me3, H3K4me1, and H3K27ac), and transcription factor occupancy (35) are shown. RPM, reads per million. Vertical bar 1 corresponds to the *SNCA* promoter, and vertical bars 2 and 3 correspond to the two enhancers. Clenbuterol (blue) and propranolol (orange) treatments modulated H3K27ac across the three regulatory sites, as determined by quantitative chromatin immunoprecipitation (ChIP) ($P < 0.05$; ANOVA with Tukey's). Dark gray, histone deacetylase inhibitor valproic acid; gray, vehicle. Means ± SEM of three independent experiments. (B) Western blotting with an antibody against H3K27ac (bottom) and relative *SNCA* mRNA levels (top) (n = 7). Means ± SEM. *P < 0.05; ** $P < 0.005$; one-way ANOVA with Tukey's. (C) Cotreatment of clenbuterol with valproic acid abrogated the β2AR agonist's effect on *SNCA* expression (green) (n = 4). Means ± SEM. * $P < 0.05$; two-tailed Student's t test.

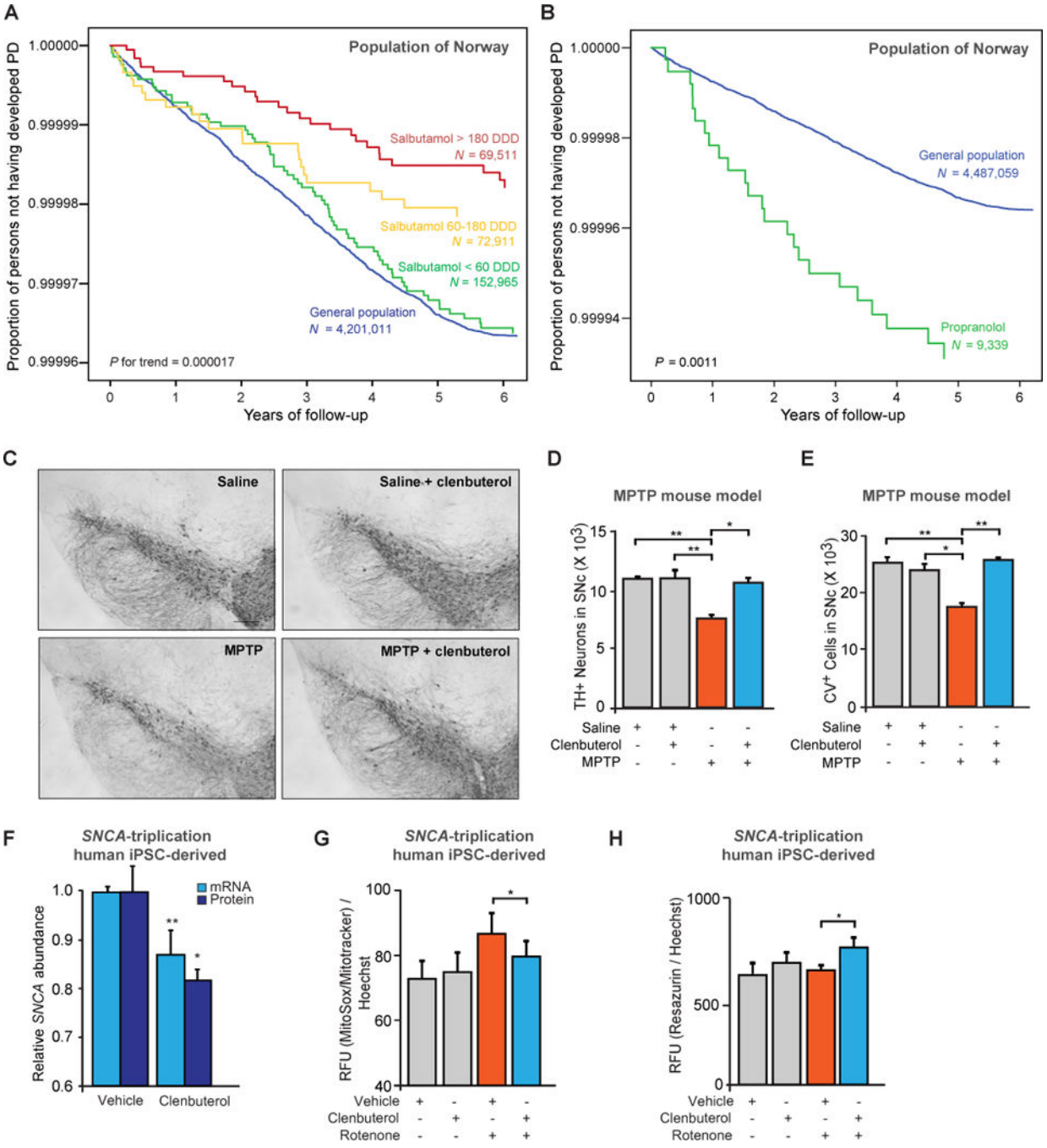


Fig. 4.

β2AR ligands are associated with risk of PD in Norway, and agonists show neuroprotective effects. (A and B) Covariate-adjusted survival curves show the proportion of individuals not developing PD from 2008 to 2014 for different exposure groups. Cox's proportional hazard regression model adjusted for age, sex, and level of education was used for these analyses. In (A), Norwegians who never were prescribed salbutamol ("never users") are represented by the blue survival curve. Individuals who were prescribed salbutamol at high [>180 defined daily doses (DDDs); red] or medium doses (60 to 180 DDDs; yellow) between 2004 and

2007 had lower proportions of incident PD during longitudinal follow-up. In (B), Norwegians who never were prescribed propranolol (“never users”) are represented by the blue survival curve. Individuals (n = 9339) who used at least 365 DDDs of propranolol between 2004 and 2007 had a higher proportion of incident PD (green) during longitudinal follow-up. (C) Representative images illustrating TH+ neurons in the substantia nigra pars compacta (SNpc). MPTP-treated animals show loss of TH+ neurons relative to control animals treated with saline or saline plus clenbuterol. Scale bar, 100 m. (D and E) Clenbuterol abrogated MPTP induced loss of nigral neurons in mice, as assayed by anti-TH immunostaining (D) or cresyl violet (CV) staining of cells (E) and stereology (n = 6 to 8 animals per group). Means ± SEM. * $P < 0.05$; ** $P < 0.01$; one-way ANOVA with Tukey’s. (F) Effect of clenbuterol treatment (20 μM) on *SNCA* mRNA expression (light blue; 3 days) and α-Syn protein expression (dark blue; 4 days) in PD patient iPSC– derived neuronal precursor cells (NPCs) carrying the *SNCA* locus triplication. Means ± SEM. * $P < 0.05$; ** $P < 0.005$; two-tailed Student’s t test. (G) Clenbuterol treatment and levels of mitochondria-associated superoxide in NPCs carrying the *SNCA* triplication. Cells were treated with or without 20 μM clenbuterol for four days and challenged with 20 μM rotenone during the last 18 hours (n = 6). (H) Clenbuterol treatment affects cellular viability of these NPCs, as determined by using resazurin, a fluorescent indicator dye of mitochondrial and other cellular reductive potentials. Cells were treated with or without 20 μM clenbuterol for 4 days and challenged with 20 μM rotenone during the last 18 hours (n = 6). RFU, relative fluorescence units. Means ± SD [(G) and (H)]. * $P < 0.05$; two-way ANOVA with Tukey’s [(G) and (H)].

Table 1

Rate ratio (RR) for Parkinson's disease in persons treated with salbutamol or propranolol during a complete 11 years follow-up of the entire population of Norway.

				RR (95% CI)	
	Users	Cases	Person-years	Age-, sex- adjusted	Multivariate adjusted[a]
Salbutamol					
Never user	4,066,119	4,398	36,700,554	1 (ref)	1 (ref)
Ever user	619,863	236	3,135,956	0.65 (0.57–0.74)	0.66 (0.58–0.76)
Propranolol					
Never user	4,671,188	4,593	39,770,912	1 (ref)	1 (ref)
Ever user[b]	14,794	41	65,598	2.16(1.59–2.94)	2.20 (1.62–3.00)

[a] Adjusted for age in 5 year periods, sex and level of education.

[b] Use of at least 365 defined daily doses.

RR: Rate ratio; PD: Parkinson's disease; Cl: confidence interval

Table 2

Rate ratio (RR) for Parkinson's disease during 2008–2014 for salbutamol prescribed during 2004–2007 in the entire population of Norway.

			RR (95% CI)
	Users 2004–07	Cases 2008–14	Multivariate Adjusted[a]
Salbutamol			
Never user	4,201,011	2,338	1 (ref)
Low (<60 DDD)	152,965	68	0.96 (0.76–1.23)
Medium (60–180 DDD)	72,911	23	0.60 (0.40–0.91)
High (≥180 DDD)	69,511	25	0.45 (0.31–0.67)

[a]Adjusted for age in five year periods, sex and level of education.

DDD: Defined daily dose; RR: Rate ratio; CI: confidence interval