

An archaeal compound as a driver of Parkinson's disease pathogenesis

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1 **An archaeal compound as a driver of Parkinson's** 2 **disease pathogenesis**

3

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38

39

40 Abstract

41 Patients with Parkinson's disease (PD) exhibit differences in their gut microbiomes
42 compared to healthy individuals¹⁻³. Although differences have most commonly been
43 described in the abundances of bacterial taxa, changes to viral and archaeal populations have
44 also been observed³⁻⁵. Mechanistic links between gut microbes and PD pathogenesis remain
45 elusive but could involve molecules that promote α -synuclein aggregation^{1,6}. Here, we show
46 that 2-hydroxypyridine (2-HP) represents a key molecule for the pathogenesis of PD. We
47 observe significantly elevated 2-HP levels in faecal samples from patients with PD or its
48 prodrome, idiopathic REM sleep behaviour disorder (iRBD), compared to healthy controls.
49 2-HP is correlated with the archaeal species *Methanobrevibacter smithii* and with genes
50 involved in methane metabolism, and it is detectable in isolate cultures of *M. smithii*. We
51 demonstrate that 2-HP is selectively toxic to transgenic α -synuclein overexpressing yeast and
52 increases α -synuclein aggregation in a yeast model as well as in human induced pluripotent
53 stem cell derived enteric neurons. It also exacerbates PD-related motor symptoms, α -
54 synuclein aggregation, and striatal degeneration when injected intrastrially in transgenic
55 mice overexpressing human α -synuclein. Our results highlight the effect of an archaeal
56 molecule in relation to the gut-brain axis, which is critical for the diagnosis, prognosis, and
57 treatment of PD.

58

59

60 Main

61 Changes to the gut microbiome have been implicated in the pathogenesis of idiopathic conditions
62 such as cancer, autoimmune, metabolic, and neurodegenerative diseases. For all, incidences have
63 been rising in the past decades⁷⁻¹¹. However, the causal mechanisms remain largely elusive, not
64 least for disorders such as Parkinson's disease (PD), where connections between distal body sites
65 (the gut) and organs classically associated with the disease (the brain) are not obvious.
66 Nevertheless, as the hub of exposures to microbiome-derived molecules, the gut represents a main
67 candidate site for the initiation of pathogenic processes which may subsequently spread
68 systemically, for example via the gut-brain axis^{6,12}.

69
70 Cross-sectional studies have highlighted microbial community differences in the gut of PD patients
71 compared to healthy control subjects^{1,3,6}. Changes in microbial abundances have also been
72 identified in patients with idiopathic rapid eye movement sleep behaviour disorder (iRBD)¹³,
73 which is recognized as a prodromal stage of α -synucleinopathies¹⁴. A smaller number of studies
74 have explored specific molecules, such as faecal short-chain fatty acids (SCFAs)^{15,16}, or used
75 multi-omic and computational approaches to identify microbial genes or functions which may be
76 enriched or depleted in PD^{4,5,17}. However, although agreement exists in relation to taxa found to
77 be enriched in PD including the genera *Akkermansia*, *Bifidobacterium* and *Lactobacillus*^{2,3}, there
78 is currently no consensus on the functional impact of these microbes on PD pathogenesis, or even
79 whether their role is causal or not.

80
81 Multi-omic approaches that provide detailed information on the functional attributes of the
82 microbiome are essential for understanding the molecular links between microbes and disease¹⁸.

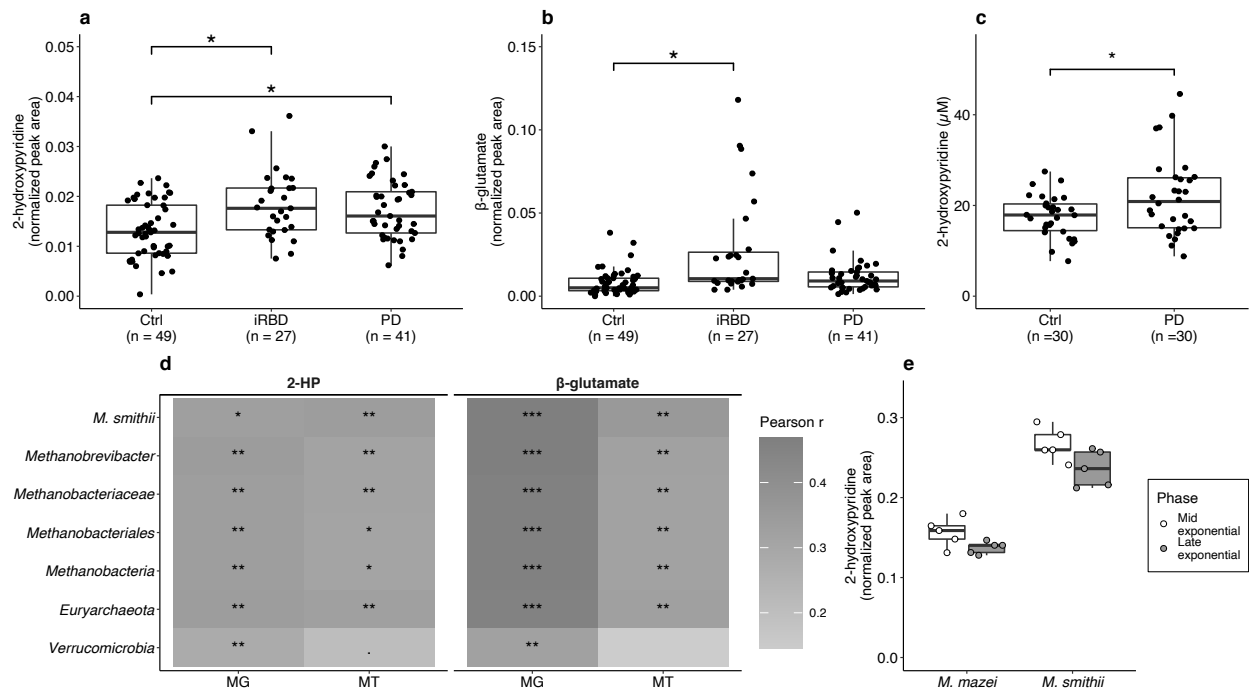
83 Here, we performed an integrated multi-omic analysis of faecal samples from PD and iRBD
84 patients and healthy control subjects to systematically investigate the functional consequences of
85 altered gut microbiota in PD. Untargeted metabolomics results revealed an initially unidentified
86 candidate molecule, which was significantly enriched in PD and iRBD and which we identified as
87 2-hydroxypyridine (2-HP; tautomer: 2-pyridone). We characterized the effects of this molecule in
88 cellular and animal models of PD pathogenesis, which uncovered an effect on relevant α -synuclein
89 aggregation, and an association with hydrogenotrophic methanogenic archaea which were
90 identified as the likely source.

91

92 **Integrated multi-omic analyses**

93 Our initial set of subjects consisted of 50 PD and 30 iRBD cases as well as 50 control subjects; 4
94 PD and 3 iRBD cases as well as 1 control subject were subsequently excluded due to reasons
95 described in the Supplementary Methods. Using our previously developed methodological
96 framework^{19,20}, we performed a systematic multi-omic analysis of DNA, RNA, proteins, and
97 metabolite fractions isolated from flash-frozen faecal samples obtained from 46 PD and 27 iRBD
98 patients as well as 49 healthy controls (Extended Data Fig 1, Extended Data Table 1a). Statistical
99 comparisons were performed for seven data types: taxonomically and functionally annotated
100 metagenomic (MG), metatranscriptomic (MT) and metaproteomic (MP) data, as well as metabolite
101 data (Extended Data Fig 2, Supplementary Tables 1-3, Supplementary Discussion). Taxonomic
102 comparisons revealed no differences in alpha diversity with any data type or metric, while beta
103 diversity differed significantly between cases (subjects with either PD or iRBD) and controls in
104 MT and MG data when adjusted for age and sex (Extended Data Fig 3a-b, Supplementary Table
105 4). Many previously reported differences between specific microbial taxa were replicated,

106 particularly in the MT data (Extended Data Fig 3c-d, Supplementary Tables 5-6). This data type
 107 also had the largest number of differentially abundant features in functionally annotated data
 108 (Extended Data Fig 3e-f, Supplementary Tables 7-8), emphasising the importance of using
 109 multiple omic datasets instead of focusing on DNA-based analyses. Metabolite comparisons
 110 highlighted significantly elevated levels of two unidentified compounds in iRBD and PD (Fig 1a-
 111 b, Extended Data Fig 4a, Supplementary Table 9). An integrated analysis of all omic datasets
 112 (Extended Data Fig 5, Supplementary Table 10) also selected these compounds among the features
 113 that best differentiate between cases and controls.



114
 115 **Figure 1: Metabolite differences between case and control subjects, and findings connecting**
 116 **differentially abundant metabolites to the gut archaeon *Methanobrevibacter smithii*.** **a.** Normalized
 117 peak area of 2-hydroxypyridine (2-HP) in faecal extracts of PD and iRBD patients and control subjects
 118 (Ctrl). **b.** Normalized peak area of β -glutamate in faecal extracts from PD and iRBD patients and control
 119 subjects. **c.** Validation of differential concentration of 2-HP in faecal extracts with targeted metabolomics.
 120 **d.** Pearson correlations of relative abundances of taxa with 2-HP and β -glutamate, trimmed to taxa with the
 121 most overlap between different analysis strategies (complete results: Extended Data Fig 4d and
 122 Supplementary Table 17); MG = metagenomic data, MT = metatranscriptomic data. **e.** 2-HP in cells of the
 123 archaeal species *Methanobrevibacter smithii* (*M. smithii*) and *Methanosarcina mazei* (*M. mazei*); five
 124 biological replicates, normalized by cell count prior to measurement. In a-c, *P*-values reflect unpaired, two-

125 tailed t-tests corrected for multiple comparisons across all metabolites; * $P < 0.05$. In all box plots: box
126 hinges: 1st and 3rd quartiles; whiskers: hinge to highest/lowest values that is within 1.5*IQR of hinge.
127

128 The first unidentified metabolite was significantly correlated with disease duration in PD patients
129 ($P=0.025$ for Pearson correlation; Extended Data Fig 4b, Supplementary Table 11). This
130 metabolite was annotated as 2-hydroxypyridine by matching its electron ionization mass spectrum
131 against publicly available mass spectral libraries (e.g. the Golm Metabolome Database²¹). Finally,
132 a commercially available chemical standard was used for definitive identification by its mass
133 spectral fingerprint and retention index. We further validated the difference in 2-HP between PD
134 patients and controls with targeted metabolomics (Fig 1c) using 60 additional faecal samples (30
135 PD, 30 controls, Extended Data Table 1b).

136
137 2-HP is a microbial degradation product of chlorpyrifos²², a pesticide known to increase the risk
138 of PD²³. We explored our faecal LC-MS/MS data for the presence of this pesticide but did not
139 detect it or related molecules (Supplementary Tables 12-16, Supplementary Discussion). In
140 comparisons with other data types (Extended Data Fig 4d-f, Supplementary Tables 17-19,
141 Supplementary Discussion), 2-HP was significantly correlated with the metagenomic (MG) and
142 metatranscriptomic (MT) abundances of archaea, specifically of *Methanobrevibacter smithii*
143 ($P=0.006$ in MT and $P=0.014$ in MG relative abundances with false discovery rate (fdr) -adjusted
144 Pearson correlation; Fig 1d, Extended Data Fig 4d, Supplementary Table 17). *M. smithii*, a
145 hydrogenotrophic methanogen, is the most abundant archaeal species in the human gastrointestinal
146 tract²⁴. It is associated with constipation²⁵, which is also a common early non-motor symptom of
147 PD²⁶. Although *M. smithii* exhibited a numerically higher mean relative abundance in PD and
148 iRBD subjects in taxon data, the difference to controls was not statistically significant in any omic

149 dataset (Extended Data Fig 6a-c, Supplementary Tables 5-6). This could be due to the overall low
150 levels of archaea in the gut, and the fact that our methodological workflow was not optimised for
151 archaeal detection. However, a significant increase in the genus *Methanobrevibacter* in PD was
152 previously reported in a meta-analysis combining 16S rRNA gene amplicon data from nine
153 studies³. Furthermore, we detected a significantly higher abundance of this genus in faecal samples
154 from an independent PD cohort from Luxembourg (NCER-PD cohort¹⁷, 343 controls and 194 PD
155 patients; Extended Data Table 1c, Extended Data Fig 6d, Supplementary Table 20). Finally,
156 considering functionally annotated MG and MT data from the main cohort of the present study, 2-
157 HP was significantly correlated with more than half of the methanogenesis-related gene functions
158 from *M. smithii* detected in data from cases, but not in data from control subjects (Extended Data
159 Fig 6e).

160

161 The second unidentified metabolite, which was particularly elevated in iRBD compared to
162 controls, was identified as β -glutamate. We found it to also be correlated with *M. smithii* relative
163 abundance (fdr-adjusted $P = 0.001$ for Pearson correlation of relative abundance in MT data and
164 $P < 0.001$ in MG data; Extended Data Fig 4d, Supplementary Table 17). β -glutamate is a known
165 archaeal osmolyte²⁷, thereby further supporting a connection between PD and archaeal
166 metabolism. 2-HP and β -glutamate were also positively correlated (Extended Data Fig 4e,
167 Supplementary Table 18). Other signals that were highly correlated with both 2-HP and β -
168 glutamate were analytes with such low abundances in the samples that structure elucidation based
169 on electron ionization mass spectrum interpretation was not possible.

170

171 We hypothesized that archaeal metabolism could be the main source of the increased 2-HP levels
172 in PD and iRBD patients' faecal samples. One potential source could be the cofactor of the enzyme
173 5,10-methenyltetrahydromethanopterin hydrogenase (Hmd), which is produced by
174 hydrogenotrophic methanogenic archaea, as this cofactor contains a 2-pyridone structure²⁸. To
175 confirm the presence of 2-HP in *M. smithii*, we measured it in cultures of the type strain of this
176 species, as well as of an environmental methanogenic archaeal species not found in the gut,
177 *Methanosarcina mazei*. 2-HP was detectable in cells of both species, with higher levels at mid-
178 exponential compared to late-exponential phase (Fig 1e).

179

180 We additionally quantified 2-HP in cerebrospinal fluid (CSF) and plasma samples from a subset
181 of subjects. It was not detectable in CSF but was detectable at low levels in plasma (Extended Data
182 Fig 4c), although concentrations did not differ between cases and controls. This indicates that 2-
183 HP can pass from the gut to circulation and could therefore trigger systemic effects beyond the
184 gut. Three different computational prediction tools²⁹⁻³¹ suggest, at high-confidence, that it can also
185 penetrate the blood-brain-barrier.

186

187 2-HP shares a substructure with the curlicide/pilicide FN075, which contains a ring-fused 2-
188 pyridone³². This compound inhibits fibrillation of the amyloidogenic curli protein CsgA, but
189 stimulates α -synuclein amyloid fibre formation³². Based on this common substructure, we
190 hypothesized that 2-HP could also influence α -synuclein aggregation, providing a potential
191 mechanistic connection to PD pathogenesis, and prompting us to study the effect of 2-HP on α -
192 synuclein aggregation and neurotoxicity.

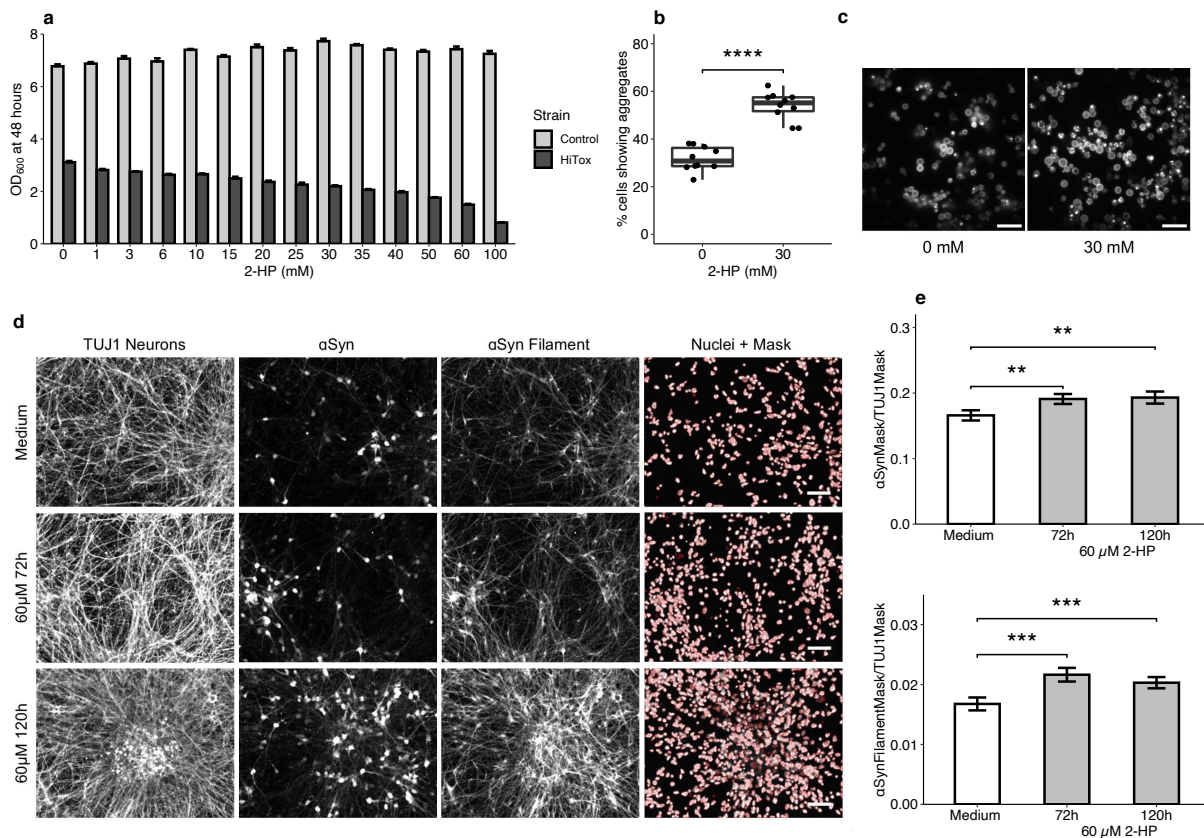
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194 **2-hydroxypyridine and α -synuclein**

195 One of the main histopathological hallmarks of PD is the formation of intracellular inclusions
196 known as Lewy bodies, the main constituent of which is α -synuclein³³. We used multiple
197 experimental approaches with appropriate concentrations, including an *in vivo* model, to explore
198 the connection between 2-HP and α -synuclein aggregation.

199
200 First, we used a yeast model overexpressing human α -synuclein, HiTox. In agreement with human
201 PD pathology, galactose-inducible expression of α -synuclein in yeast leads to cytosolic α -
202 synuclein aggregation and severe growth defects by disrupting vesicle trafficking, mitochondrial
203 function and lipid homeostasis³⁴⁻³⁷. These phenotypes have been translated in human-derived cell
204 models, demonstrating the strength of this model to study cellular α -synuclein-induced
205 cytotoxicity^{37,38}. Based on preliminary testing, micromolar concentrations had no detectable
206 effects, and a range of 1 mM to 100 mM was chosen for the subsequent experiments. We confirmed
207 that at these levels, 2-HP exacerbates α -synuclein-induced toxicity in the HiTox model in a dose-
208 dependent manner (Fig 2a, Extended Data Fig 7a). Growth defects were already seen at 1 mM,
209 and a 100 mM concentration was almost lethal. In contrast, the control strain, which does not
210 express human α -synuclein, showed a slight growth improvement in the presence of higher doses
211 of 2-HP. When α -synuclein expression was not induced, both strains showed only a mild growth
212 defect at 100 mM (Extended Data Fig 7a-b), confirming that 2-HP is toxic only when the strain
213 expresses human α -synuclein. We additionally tested two related compounds, namely 3-
214 hydroxypyridine (3-HP) and 4-hydroxypyridine (4-HP). 4-HP had only a mild effect on the HiTox
215 strain at 30 mM and above, while 3-HP was very toxic to both strains at concentrations above 10
216 mM (Extended Data Fig 7c). Based on this, 2-HP has the clearest dose-response effect when

217 compared to the two other hydroxypyridine isomers. Furthermore, microscopic inspection of the
 218 HiTox strain under moderately toxic conditions (30 mM 2-HP) revealed a significant increase in
 219 cells with cytosolic α -synuclein aggregates in comparison to the control conditions (Fig 2b-c).
 220 Taken together, our results demonstrate that 2-HP stimulates α -synuclein aggregation and
 221 exacerbates its cytotoxic effect in the HiTox yeast model.
 222



223
 224 **Figure 2. Effects of 2-hydroxypyridine in human α -synuclein expressing yeast model and in human**
 225 **induced pluripotent stem cell (hiPSC) derived enteric neurons. a.** Dose-response assay in α -synuclein
 226 expressing yeast cells (HiTox) and control strain (Ctrl); showing means and standard deviations calculated
 227 from four biological replicates for each strain. **b.** Microscopy-based quantification of yeast cells exhibiting
 228 α -synuclein aggregates (based on 10 pictures per condition). Box hinges: 1st and 3rd quartiles; whiskers:
 229 hinge to highest/lowest values that is within 1.5*IQR of hinge. **c.** Representative microscopy images of
 230 HiTox cells after 24 h treatment with 30 mM 2-HP; scale bar: 20 μ m. **d.** High-content imaging of hiPSC
 231 derived enteric neurons, showing total α -synuclein (α Syn), α -synuclein filament (α Syn Filament), TUJ1-
 232 positive neurons and Hoechst-positive nuclei (with a representation of the mask applied to segment the

233 nuclei); scale bar: 100 μ m. **e.** Total α -synuclein and filamentous α -synuclein normalized to the amounts of
234 TUJ1-positive neurons (mean \pm SEM of three independent neuronal differentiations; 8 wells per condition
235 and 30 fields per well quantified for each). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

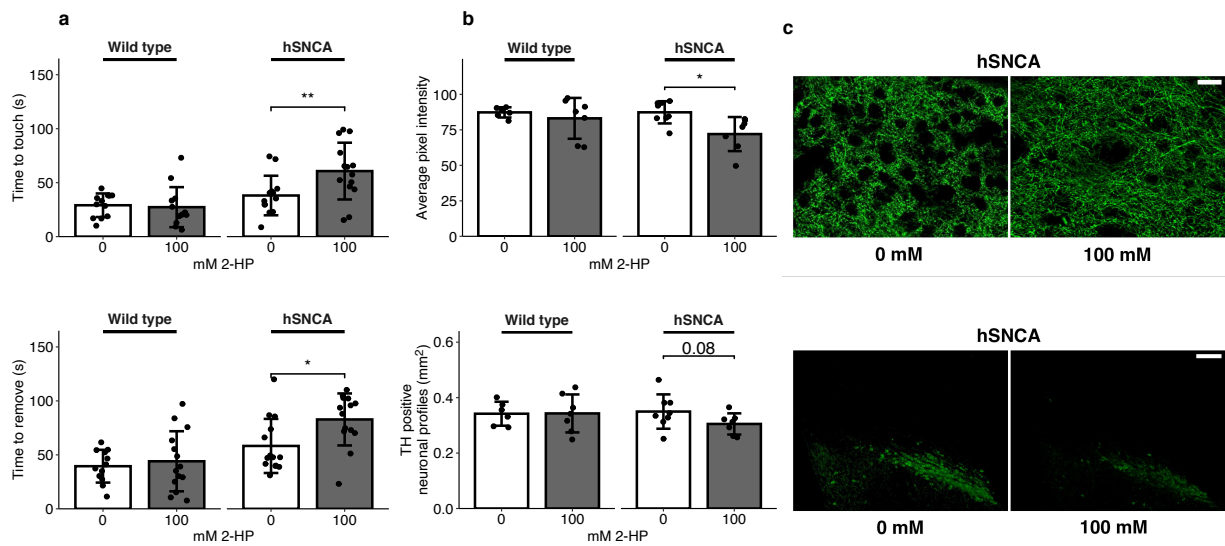
236

237 To further evaluate the toxicity of 2-HP in human induced pluripotent stem cell (iPSC)-derived
238 enteric nervous system (ENS) neurons³⁹ (Extended Data Fig 8a-b), we treated ENS cells with 60
239 μ M 2-HP, the highest concentration that did not severely impair cell viability in preliminary dose
240 tests. After 120 hours of exposure, we did not observe a significant decrease in the viability of the
241 cultures (Extended Data Fig 8c). However, we observed a significant increase in both total and
242 fibrillated α -synuclein (Fig 2d-e) with a concomitant increase in the apoptotic marker cleaved-
243 caspase 3 (CC3) (Extended Data Fig 8d-e), supporting that exposure to 2-HP also drives α -
244 synuclein accumulation in ENS neurons.

245

246 Finally, we explored the effects of 2-HP on the central nervous system (CNS) *in vivo* using an
247 established experimental approach for studying the effect of α -synuclein toxicity-promoting agents
248 in mice^{40,41}. We performed intrastriatal injections of 100 mM 2-HP into a mouse model
249 overexpressing human wild-type *SNCA* (hSNCA) under the transcriptional control of the neuronal
250 *Thy1* promoter, and wild-type littermate controls. The concentration was selected based on
251 previous literature of neurodegeneration-inducing molecules for which the dose used in stereotaxic
252 injections *in vivo* is typically 100 x higher than the one used *in vitro*⁴². The mice were all
253 heterozygous for the transgene, males, and 2-3 months old. Two months after the injection, we
254 observed that fine motor skills, assessed with the adhesive removal test⁴³, were significantly
255 impaired in hSNCA mice injected with 2-HP compared to those injected with vehicle solution
256 without 2-HP (Fig 3a). To determine the pathological basis of the impairment, we measured the

257 relative levels of tyrosine-hydroxylase (TH), a marker for dopaminergic neurons and their
 258 projecting fibres into the dorsal striatum⁴⁰. We found that TH was significantly reduced in the
 259 dorsal striatum, and somewhat reduced in the substantia nigra pars compacta (SNpc) in the 2-HP
 260 injected hSNCA mice (Fig 3b-c). We also measured S129 phosphorylated α -synuclein (phospho-
 261 α Syn), the most commonly used marker to identify α -synuclein inclusions⁴⁴, in synaptic boutons
 262 (striatum, substantia nigra) and in cell body profiles in different brain regions of the mice in our
 263 study. We found a decrease of phospho- α Syn in the dorsal striatum of 2-HP-injected transgenic
 264 mice, possibly reflecting the structural neuronal injury induced in this region by 2-HP (Extended
 265 Data Fig 9a). We observed no significant effect in the SNpc, where a longer timespan between
 266 injection and analysis may be necessary to elicit an effect (Extended Data Fig 9b). Interestingly,
 267 in the prefrontal cortex, a brain region neuronally connected to the dorsal striatum, we observed a
 268 significant increase in the number of intracellular α -synuclein inclusions in 2-HP injected
 269 transgenic mice compared to their control counterparts (Extended Data Fig 9c).



270

271 **Figure 3. Effects of intrastriatally injected 2-hydroxypyridine on fine motor behaviour and the**
 272 **dopaminergic neuron marker tyrosine hydroxylase (TH) in transgenic mice overexpressing human**
 273 **α -synuclein. a.** Fine motor behaviour of mice 2 months after 2-HP injection, measured using the adhesive
 274 removal test (time to touch: upper panel, time to remove: lower panel); hSNCA: human α -synuclein

275 overexpressing transgenic mice; n of mice = 12-14 per group. **b.** Quantification of TH-positive structures
276 in the nigro-striatal circuit of hSNCA mice at 2 months after 100 mM 2-HP injection; Upper panel: TH-
277 positive axons in the dorsal striatum; lower panel: TH-positive neurons in the substantia nigra pars
278 compacta; n of mice = 6-8 per group. **c.** Microphotographs showing examples for 0 mM and 100mM in
279 hSNCA mice only; upper panel: dorsal striatum (scale bar: 25 μ m); lower panel: substantia nigra pars
280 compacta (scale bar: 200 μ m). All bar plots show mean and standard deviation. * $P < 0.05$, ** $P < 0.01$.

281

282 **Discussion**

283 In mammals, 2-HP is a known metabolite of pyridine⁴⁵. While pyridine ring structures are
284 commonly present in biological systems and in man-made compounds, such as drugs and
285 pesticides, unsubstituted pyridine is not common in nature⁴⁶. We initially hypothesized that the 2-
286 HP we measured in faecal samples could originate from bacterial breakdown of the pesticide
287 chlorpyrifos, where 2-HP is an established intermediate product²². However, we did not detect
288 chlorpyrifos or other related compounds in our faecal metabolomic data. Instead, our results
289 indicate an association of 2-HP and the archaeal species *Methanobrevibacter smithii* as well as
290 methanogenesis-related genes on the metagenomic and metatranscriptomic level. Moreover, we
291 found β -glutamate, which is also linked to archaeal metabolism²⁷ to be statistically significantly
292 different between PD, iRBD, and healthy control samples. At present, there is no established
293 biosynthetic pathway for 2-HP in *Methanobrevibacter smithii* or in any other organism, but many
294 hydrogenotrophic methanogenic archaea produce a cofactor which has a 2-pyridone structure²⁸.
295 We detected 2-HP in methanoarchaeal cells, suggesting that there is a metabolic process that
296 produces this compound.

297

298 Our results show that 2-HP has effects relevant to PD pathogenesis in several model systems:
299 increased α -synuclein aggregation in a humanized yeast model and in human iPSC-derived enteric
300 neurons and exacerbated behavioural symptoms and neuropathological changes in a mouse model

301 of PD. 2-HP has not been widely studied in mammals previously, and there are no reported
302 neurotoxic effects thus far. However, a ring-fused 2-pyridone compound (FN075) was shown to
303 increase α -synuclein aggregation through a mechanism that involves the formation of oligomers
304 with a flexible solvent-exposed C-terminal end; these oligomers then drive α -synuclein
305 fibrillation³². Injection of FN075 into the striatum or the substantia nigra of mice also causes PD-
306 like behavioural, metabolic and neuropathological changes, and these changes are absent in mice
307 that do not produce α -synuclein⁴⁷. An analogous mechanism could explain our *in vitro* and *in vivo*
308 findings concerning 2-HP and α -synuclein.

309

310 We detected 2-HP at highest concentrations in faecal samples compared to plasma or CSF,
311 implicating the gut as a likely anatomic origin. This is in line with Braak's dual hit hypothesis¹²,
312 which posits that in PD α -synuclein aggregation begins in the gut and spreads from the enteric
313 nervous system to the central nervous system. Furthermore, the presence of 2-HP in plasma,
314 combined with our prediction that it can likely pass the blood-brain barrier, represents another
315 potential route for spreading. We hypothesize that 2-HP could act together with other PD-
316 influencing microbial mechanisms. This would be parallel to the gut microbiome-dependent
317 process which has been described in mouse models of PD using the pesticide/insecticide rotenone,
318 administered via intrastriatal injections^{48,49}. For example, intestinal barrier disruption due to mucus
319 erosion by *Akkermansia muciniphila*⁵⁰, a microbe that is often reported to be increased in PD
320 patients^{3,13}, could lead to increased exposure to harmful microbial metabolites, such as 2-HP
321 produced by *M. smithii*. This would then drive α -synuclein aggregation, initiating or exacerbating
322 PD pathogenesis. In this context, it has not escaped our attention that a mechanism involving *M.*

323 *smithii* represents a first known example for a clear role for archaea in a human disease
324 pathogenesis.

325

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443

444 Methods

445 Patient cohorts and sampling

446 Individuals with PD (n = 50) or iRBD (n = 30) and healthy control subjects (n = 50) were recruited
447 from two study sites in Germany (Paracelsus-Elena Klinik, Kassel, and Philipps-University,
448 Marburg). The studies conformed to the Declaration of Helsinki and the ethical guidelines of the
449 respective institutions (Kassel: approved by the ethics committee of the Physician’s Board Hessen,
450 Germany (FF 89/2008), and the DeNoPa trial registered at the German Register for Clinical trials
451 (DRKS00000540); Marburg: approved by the ethics committee of the Medical Faculty of the
452 Philipps-University, Marburg, Germany (46/14)). All subjects provided informed written consent.
453 The sample analysis was approved by the Comité National d’Ethique de Recherche of
454 Luxembourg (reference no.: 140174_ND). For the targeted validation measurements of our main
455 metabolomics finding, we used a set of DeNoPa cohort samples consisting of 25 later-time point
456 samples from individuals included in the main multi-omic analyses, and 35 samples from

457 previously unmeasured individuals. Further details on recruitment, inclusion and exclusion criteria
458 and clinical data collection are provided in the Supplementary .

459

460 For both cohorts, faecal samples were collected at the clinic via a stool specimen collector
461 (MedAuxil) and collection tubes (Sarstedt), as previously described¹³. Samples were immediately
462 flash-frozen on dry ice after collection. Samples were subsequently stored at -80°C and shipped
463 on dry ice. For the Kassel cohort, blood and cerebrospinal fluid samples were also obtained, as
464 described previously⁵¹.

465

466 Extractions from faecal samples were performed according to a previously published protocol^{19,52},
467 conducted on a customized robotic system (Tecan Freedom EVO 200). After exclusions, the final
468 sample set consisted of samples from 46 PD and 27 iRBD cases and 49 control subjects; see
469 Supplementary for additional details.

470

471 **Metagenomic and metatranscriptomic sequencing**

472 For metagenomics, all DNA samples were subjected to random shotgun sequencing. Following
473 DNA isolation, 200-300 ng of DNA was sheared using a Bioruptor NGS (Diagenode) with 30s
474 ON and 30s OFF for 20 cycles. Sequencing libraries were prepared using the TruSeq Nano DNA
475 library preparation kit (Illumina) following the manufacturer's protocol, with 350 bp average insert
476 size.

477

478 For metatranscriptomics, 1 μg of isolated RNA was rRNA-depleted using the RiboZero kit
479 (Illumina, MRZB12424). Library preparation was performed using the TruSeq Stranded mRNA

480 library preparation kit (Illumina) following the manufacturer's protocol, apart from omitting the
481 initial steps for mRNA pull down.

482

483 For metagenomic and metatranscriptomic analyses, the qualities of the libraries were checked
484 using a Bioanalyzer (Agilent) and quantified using Qubit (Invitrogen). Libraries were sequenced
485 on an Illumina NextSeq500 instrument with 2x150 bp read length.

486

487 **Metaproteomics**

488 Following isolation, 20 µl of protein extracts were processed using the paramagnetic bead
489 approach with SP3 carboxylate coated beads^{53,54}; further details are provided in the Supplementary
490 . The resulting peptide lysates were analysed on a Q Exactive HF instrument (Thermo Fisher
491 Scientific) equipped with a TriVersa NanoMate source (Advion) in LC chip coupling mode.
492 Peptide lysates were injected on a trapping column (Acclaim PepMap 100 C18, 3 µm, nanoViper,
493 75 µm x 2 cm, Thermo Fisher Scientific) with 5 µL/min by using 98% water/2% ACN 0.5%
494 trifluoroacetic acid, and separated on an analytical column (Acclaim PepMap 100 C18, 3 µm,
495 nanoViper, 75 µm x 25 cm, Thermo Fisher Scientific) with a flow rate of 300 nl/min. The mobile
496 phase was comprised of 0.1% formic acid in water (A) and 80 % ACN/0.08 % formic acid in water
497 (B). Full MS spectra (350–1,550 m/z) were acquired in the Orbitrap at a resolution of 120,000 with
498 automatic gain control (AGC) and a target value of 3×10^6 ions.

499

500 **Metabolomics**

501 Untargeted GC-MS and SCFA measurements from faecal samples were performed according to a
502 previously published protocol⁵². Details for untargeted LC-MS/MS measurements from faecal
503 samples are provided in the Supplementary .

504

505 For targeted 2-HP detection in faecal samples, polar metabolites were extracted as follows: 500
506 μ L of MilliQ water was added to 50 mg of faecal matter. Samples were homogenized using a
507 Precellys24 homogenizer (Bertin Technologies): 6000 rpm, 1x30 s at 0 to 5 °C. Plasma and CSF
508 as well as further sample preparation of samples and measurement parameters were performed as
509 previously described⁵⁵. For unambiguous identification and precise quantification of 2-HP,
510 concentrations were determined using a standard addition approach. Aliquots of the same sample
511 (faecal sample, plasma or CSF, with added internal standards: pentanedioic acid-D6, [U-¹³C]-
512 ribitol and tridecanoic acid-D25) were separately spiked with different concentrations of 2-HP (10,
513 50, 100, 150 and 200 μ mol/l) to extrapolate compound levels. Derivatization and GC-MS
514 measurements were performed as reported before.

515

516 All GC-MS chromatograms were processed using MetaboliteDetector, v3.220190704⁵⁶.
517 Compounds were initially annotated by retention time and mass spectrum using an in-house mass
518 spectral library. Internal standards were added at the same concentration to every medium sample
519 to correct for uncontrolled sample losses and analyte degradation during metabolite extraction.
520 The data was normalized by using the response ratio of the integrated peak area of the analyte and
521 the integrated peak area of the internal standard.

522

523 **Bioinformatics and statistics for multi-omics**

524 Metagenomic and metatranscriptomic sequencing data were analysed using the IMP pipeline,
525 version 01.07.2020 (<https://git-r3lab.uni.lu/IMP/imp3>, tag 6f1badf7), using the HPC facilities⁵⁷ of
526 the University of Luxembourg. Metagenomic and metatranscriptomic reads were quality-
527 controlled and co-assembled, ORFs predicted, reads and contigs taxonomically annotated, and
528 ORFs functionally annotated as previously described⁵⁸. Protein libraries were generated from the
529 IMP output and used for protein identification on a per sample basis using SearchGUI⁵⁹ (v. 3.3.20).
530 Further details are provided in the Supplementary .

531
532 All statistical analyses and visualizations were performed in R⁶⁰ (v. 4.1.0) using targets⁶¹ (v. 0.8.1)
533 for workflow management and knitr⁶²⁻⁶⁴ (v. 1.36) for reporting. Unless otherwise specified, false
534 discovery rates were used for multiple comparison correction. Differential abundance comparisons
535 were performed with DESeq2⁶⁵ (v. 1.32.0; adjusted for age and sex) for metagenomic and
536 metatranscriptomic data, and with ANOVA (adjusted for age and sex) and two-sided t-tests (not
537 corrected for confounders) for the metabolomic and metaproteomic data. Integrated multi-omic
538 testing was implemented with the DIABLO workflow from mixOmics⁶⁶ (v. 6.17.29). Additional
539 details are provided in the Supplementary .

540

541 **2-HP detection in methanoarchaeal cultures**

542 The archaea *Methanosarcina mazei* (DSM #3647) and *Methanobrevibacter smithii* (DSM #861)
543 were cultivated under anaerobic conditions in closed serum bottles containing 50 mL minimal
544 medium complemented with 1 mM sodium sulphide, 2 mM cysteine and ampicillin to avoid

545 bacterial contamination⁶⁷. As carbon and energy sources, 150 mM methanol for *M. mazei* and
546 H₂/CO₂ (80:20) in the gas atmosphere for *M. smithii* were added. Cultures were incubated at 37
547 °C. For monitoring growth, the turbidity at 600 nm was measured using an Ultraspec 2100 Pro
548 Photometer (Amersham Biosciences). Cell numbers were determined in parallel using a Thoma
549 cell counting chamber. Cells were harvested in mid and late exponential phase by centrifugation
550 at 2,455 x g for 20 min at 4 °C. The cell pellets were resuspended in 0.9 % sodium chloride solution
551 and centrifuged again (21,130 x g, 10 min, 4°C). All samples were stored at -80 °C. 2-HP was
552 measured using targeted GC-MS, with sample quantities normalized for cell counts; full details
553 are provided in the Supplementary .

554

555 **Yeast model**

556 The human *SNCA* gene was cloned into pAG306GAL-ccdB-EGFP and pAG304GAL-ccdB-EGFP
557 plasmids using the Gateway cloning system⁶⁸. Yeast cells were transformed with the constructs
558 using the standard polyethylene glycol/lithium acetate protocol⁶⁹. The *PDR5* gene was
559 subsequently replaced by *URA3* marker in the HiTox and control strains followed by selection in
560 synthetic complement (SC) media lacking uracil using the same transformation protocol⁶⁹. Gene
561 integration or disruption was verified by PCR. Strain details are provided in the Supplementary .

562

563 Four fresh single colonies of the HiTox strain and its respective control strain were inoculated
564 from SC-2% glucose plates into 5 mL SC-2% raffinose (SCR) and incubated overnight with
565 shaking (200 rpm) at 30 °C for 20 h. Cultures were subsequently diluted to OD 0.5 and 2 µL of
566 culture was added to 78 µL 2-HP containing media to a final optical density of 0.0125 in a 384-
567 well microplate. 2-HP was diluted in SC-2% raffinose/galactose (SCR/SCG) and tested at different

568 concentrations (1-100 mM). Finally, plates were measured in a microplate reader (TECAN™
569 Infinite M200Pro), at an interval of 10 minutes during 72 h at 30 °C. Yeast growth phenotyping
570 was performed as previously described⁷⁴. For better comparability between batches, strains and
571 methods, the final biomass was corrected using the GATHODE software⁷⁰. The OD600 at 48 h
572 was recorded for biomass quantification, with the means and standard deviations calculated from
573 four biological replicates.

574

575 To evaluate α -synuclein aggregation, one single colony was inoculated in 5 mL SCR (α -syn 'off')
576 and incubated at 30 °C overnight. The following day, 1 mL of culture was transferred into an
577 sample tube and cells were centrifuged. Pellets were resuspended with SCG (α -syn 'on') in
578 presence or absence of 30 mM 2-HP and incubated at 30 °C. After 24 h, cells were visualized using
579 a Nikon Microscope (100x oil). Ten pictures per condition were randomly taken. The total number
580 of cells and cells showing aggregates were manually quantified in ImageJ. Statistical significance
581 between conditions was determined using an unpaired two-sided t-test in R.

582

583 **Enteric neuron model**

584 Enteric neurons were derived from human induced pluripotent stem cells (hiPSCs) following a
585 previously published protocol³⁹; the full details are available in the Supplementary Methods.

586

587 For cytotoxicity testing, enteric neurons were detached with accutase (Sigma) after 21 days of
588 culturing in 6-well plates. The cells were replated into 96-well plates coated with poly-
589 ornithine/laminin/fibronectin as described in the Supplementary methods. They were then
590 maintained under differentiating conditions until day 31, when they were treated with 2-HP

591 (Sigma,) at 1 μ M, 3 μ M, 6 μ M, 10 μ M, 30 μ M, 60 μ M, 100 μ M, 300 μ M, 600 μ M, 1 mM and 3
592 mM. 2-HP was reconstituted to 10 mM in the differentiation medium. Cells were treated for 24h,
593 then assessed using a tetrazolium assay for viability; see Supplementary Methods for details.

594

595 For immunostaining, enteric neurons were replated into 96-well imaging plates (Cell Carrier Ultra,
596 Perkin Elmer) after three weeks in culture. Cells were maintained under differentiating conditions
597 until day 31, when they were treated with 60 μ M 2-HP for 72 h and 120 h. After treatment, cells
598 were immunostained with α -synuclein antibody (NOVUS biologicals, NBP1-05194, 1:1000), α -
599 synuclein filament antibody (Abcam, ab20953, 1:5000), TUJ1 (Millipore, AB9354, 1:600) and
600 cleaved caspase-3 (Asp175) antibody (CST, 9661, 1:200) and imaged to quantify each marker (full
601 details in the Supplementary Methods). Differences were evaluated using one-way ANOVA with
602 the mean values of three independent replicates and a two-sided Dunn's multiple comparison test
603 at each time point versus the untreated using Graphpad Prism (v. 9).

604

605 **Mouse model**

606 A transgenic mouse line, B6.D2-Tg(Thy1-SNCA)14Pjk (Line 14), which overexpresses human
607 wildtype α -synuclein under the transcriptional control of the Thy1 promoter, was used^{71,72}.
608 Genotyping of the mice is described in the Supplementary Methods. The mice were all
609 heterozygous for the transgene, males, and 2-3 months old. Between 6 and 14 mice were used for
610 each analysis. Mice had access to food and water *ad libitum* and were exposed to a regular 12h-
611 day-night cycle. Mice were injected intracranially with 100 mM 2-HP (Sigma), or PBS vehicle
612 solution (control mice), in volumes of 2 μ L, within the right dorsal striatum; full details of the
613 injection protocol are provided in the Supplementary Methods.

614

615 At two months post-injection, motor function was evaluated with the adhesive removal test⁴³.
616 Briefly, animals were placed in a round transparent arena for one minute for habituation.
617 Rectangular white tape, 3 x 5 mm was placed on the left forepaw (expected to be affected by
618 injection of 2-HP into the right dorsal striatum). The time intervals to first touch and removal of
619 the tape were recorded. The test was performed twice, sequentially, for each mouse, and times for
620 both measures were averaged. The sequence of the mice being tested was randomized, and the
621 experimenter was blinded to their genotype and treatment.

622

623 After behavioural evaluation, the mice were deeply anaesthetised (i.p. injection of medetomidine,
624 1mg/kg and ketamine, 100 mg/kg) and then euthanised by transcardial perfusion with PBS. Brains
625 were removed from the skull and post-fixed in fresh phosphate-buffered 4% paraformaldehyde for
626 48 h at 4 °C, then stored in PBS with 0.05% sodium azide (as a preservative) at 4 °C, before being
627 processed for immunofluorescence analysis.

628

629 Immunofluorescent stainings on 50 µm sections, generated with a Leica VT 100 vibratome, were
630 performed following a standard protocol⁷³. Briefly, sections were first washed in PBS with 0.1%
631 Triton X100 (T_{X100}). They were subsequently treated with a permeabilization solution (PBS +
632 1.5% T_{X100} + 3% H₂O₂) for 30 minutes. This was followed by washing with PBS + 0.1% T_{X100}.
633 To prevent unspecific antibody binding, the sections were then incubated for 1 h in PBS + 0.1%
634 T_{X100} with 5% BSA. After one short washing step, sections were incubated with the first antibodies
635 diluted in antibody solution (PBS + 0.1% T_{X100} + 2% BSA) overnight at room temperature (RT)
636 on an orbital shaker. The following antibodies were used: anti- α -synuclein (aSyn, phosphorylated

637 at S129; Prothena Biosciences Inc., 11A5; 1:1000), anti-tyrosine hydroxylase (TH; Abcam,
638 ab76442/Merck (Sigma-Aldrich), AB152; 1:1000). The sections were double-stained for TH and
639 S129 phosphorylated α -synuclein (phospho- α Syn). The next day, sections were washed with PBS
640 + 0.1% T_{X100} to remove any excess of the first antibody. Sections were then incubated with a
641 secondary antibody in antibody solution for 2 h at RT on an orbital shaker. Finally, sections were
642 washed with PBS + 0.1% T_{X100} (at least 3 times for 10 mins), then mounted on Superfrost™
643 (ThermoFisher Scientific) slides, left to dry, and finally covered with a cover-slip using
644 Fluoromount-G® (Invitrogen).

645

646 Imaging of the sections was performed using a Zeiss AxioImager Z1 upright microscope, coupled
647 to a “Colibri” LED system, and an Mrm3 digital camera for image capture using the Zeiss ZEN 2
648 Blue software. Measurements were performed on blinded sections, and codes were broken only
649 after all measurements were completed. TH-positive signals were quantified in the dorsal striatum
650 and substantia nigra pars compacta, and phospho- α Syn in these two regions as well as the
651 prefrontal cortex; details of the quantifications for each region and antibody are given in the
652 Supplementary Methods.

653

654 Behaviour and neuropathology data were analysed with Graphpad Prism (v. 9). All datasets passed
655 normality tests and were analysed by ANOVA followed by Tukey’s or Dunn’s post hoc tests.

656

657 The animal study was approved by the University of Luxembourg Animal Experimentation Ethics
658 Committee (LUPA 2020/26) and the overseeing Luxembourg Government authorities (Ministry
659 of Health and Ministry of Agriculture).

660 Data availability

661 The datasets generated by this study are available in the following repositories: metagenomic and
662 metatranscriptomic data at the NCBI BioProject collection with the ID PRJNA782492
663 (<http://www.ncbi.nlm.nih.gov/bioproject/782492>), metaproteomic data at the Proteomics
664 Identifications (PRIDE) database with accession number PXD031457
665 (<https://www.ebi.ac.uk/pride/archive/projects/PXD031457>), and metabolomic data at
666 MetaboLights with ID MTBLS5092 (<https://www.ebi.ac.uk/metabolights/MTBLS5092>). Due to
667 privacy restrictions, clinical and demographic data are available on request from the corresponding
668 author. NCER-PD clinical and 16S rRNA amplicon sequencing data are available on request from
669 <https://www.parkinson.lu/research-participation>.

670

671 Code availability

672 The IMP pipeline, which was used for analysis of metagenomic and metatranscriptomic data, is
673 available at <https://gitlab.lcsb.uni.lu/IMP/imp3>. The R code used for statistical analyses and
674 visualisations is available at <https://gitlab.lcsb.uni.lu/ESB/mibipa-2-hp>.

675

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769

770 Ethics declarations

771 **Competing interests**

772 J.-P.T. and P.W. are inventors in patent applications involving the discoveries described in this
773 publication: 1) No. LU101477, No. PCT/EP2020/081855, EP20820786.0, US17/776,001,
774 CA3,157,474; 2) No. LU101476, No. PCT/EP2020/081832, EP20820785.2, US17/776,010, filed
775 by the University of Luxembourg. V.T.E.A. is an inventor in the following patents which concern
776 the use of microbes in the diagnosis and treatment of Parkinson's disease: FI127671B,
777 EP3149205B1 and US10139408B2 (issued); US20190137493A1, US20210109098A1 and
778 EP3789501A1 (pending); these patents are currently assigned to NeuroBiome Ltd. W.O. is the
779 president of the European Brain Council. P.W. and B.M. are members of the scientific steering
780 committee for a clinical trial by 4D Pharma. The rest of the authors declare no competing interests.

781

782 Additional information

783 **Supplementary information**

784 Supplementary information is available for this paper.

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788 **Extended data figures and tables**

789 **Extended Data Figure 1.** Multi-omic data analysis workflow. PD = Parkinson's disease, iRBD =
790 idiopathic REM sleep behaviour disorder, Ctrl = control subject.

791 **Extended Data Table 1. Demographic and clinical data on study subjects.** a. Main multi-omic
792 analyses, b. 2-HP targeted validation, c. NCER-PD cohort (16S rRNA gene amplicons).

793 **Extended Data Figure 2. Independent Principal Component Analysis (IPCA) for each data**
794 **type.** Testing was performed separately for each data type, with total number of components
795 chosen based on kurtosis. The first two components are shown for each data type regardless of
796 total number of components. In the 1st column, green triangles and dashed line correspond to
797 control subjects, and black circles and solid line to patients with PD or iRBD. Ellipses reflect 95%
798 confidence. 2nd and 3rd columns show 10 features with the highest loadings. a. Untargeted
799 metabolomics; showing 2/2 components, b. Taxonomically classified metagenomic reads; 2/3
800 components, c. Taxonomically classified metatranscriptomic reads; 2/2 components, d.
801 Taxonomically classified metaproteomic spectra; 2/4 components, e. Functionally classified
802 (KEGG orthologs; KOs) metagenomic reads; 2/3 components, f. Functionally classified (KOs)
803 metatranscriptomic reads; 2/4 components, g. Functionally classified (KOs) metaproteomic
804 spectra; 2/4 components. The numbers in taxon names (b, c) are identifiers from the mOTUs
805 workflow. For KOs (e-g), details are provided in Supplementary Table 3.

806 **Extended Data Figure 3. Microbial diversity and differential abundance comparisons of**
807 **taxonomic and functional data.** a. Alpha diversity and group in taxonomically classified
808 metagenomic (MG) and metatranscriptomic (MT) sequence reads; box hinges: 1st and 3rd
809 quartiles; whiskers: hinge to highest/lowest values that is within 1.5*IQR of hinge; gray cross:
810 mean, b. Beta diversity and group (Non-metric Multidimensional Scaling based on Bray-Curtis
811 dissimilarity) in MG and MT species data, with ellipses showing 95% confidence, c. Number of
812 differentially abundant taxa (q (false discovery rate adjusted P) < 0.05); MG and MT data tested
813 with DESeq2 and MP with ANOVA (age and sex adjusted) and two-sided t-tests, d. Genera with
814 $q < 0.05$ in MG or MT data for the two-group case (either PD or iRBD) vs control contrast, e.
815 Number of differentially abundant ($q < 0.05$) KEGG orthologs (KOs) in MG, MT, and
816 metaproteomic (MP) data;); MG and MT data tested with DESeq2 and MP with ANOVA (age
817 and sex adjusted) and two-sided t-tests, e. 10 pathways most commonly represented by the
818 differentially abundant KOs ($q < 0.05$) for the case vs control comparison in MG, MT and MP
819 data. In heatmaps, . $0.1 > q > 0.05$, * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.

820 **Extended Data Figure 4. Additional results for metabolomics.** a. Metabolites with q (false
821 discovery rate adjusted P) < 0.05 for the two-group case (PD or iRBD) vs control contrast and at
822 least one pair of three-category groups, b. 2-HP normalized peak area and disease duration in PD
823 patients; grey values = full data, black values = two longest-duration subjects excluded, c. 2-HP in
824 plasma (targeted metabolomics); 2 extreme outliers excluded (2-HP concentration $> 30 \mu\text{M}$, both
825 in the iRBD group); box hinges: 1st and 3rd quartiles; whiskers: hinge to highest/lowest values
826 that is within 1.5*IQR of hinge; grey cross: mean, d. Correlations of metabolites and taxa,
827 showing taxa with $q < 0.05$ in more than 20 comparisons out of a total of 32 approaches: 2
828 metabolites, 2 data types (MG or MT), 2 annotation methods (Kraken or mOTUs), 2

829 normalizations (RA = relative abundance, CLR = centered log ratio transform) and 2 correlation
830 coefficients (Pearson or Spearman), e. Correlations of 2-HP and β -glutamate and other metabolites,
831 showing top 5 by q in full data for each metabolite and correlation coefficient, f. 10 most common
832 pathways represented by KEGG orthologs significantly correlated with 2-HP ($q < 0.05$, and for
833 MG and MT data, absolute value of correlation coefficient > 0.4). In all plots, MG = metagenomic
834 data, MT = metatranscriptomic data, MP = metaproteomic data. In plots with stars, $. 0.1 > q >$
835 0.05 , * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.

836 **Extended Data Figure 5. Results of integrated multi-omics with DIABLO.** The classification
837 was run for the two-category case (iRBD or PD) vs control variable, selecting the 5 best features
838 for differentiating between these categories on 2 axes for each of the seven data types (MG =
839 metagenomic, MT = metatranscriptomic, MP = metaproteomic; KOs: functional annotations as
840 represented by KEGG orthologs). a. Design matrix used for DIABLO, calculated based on running
841 PLS for each pair of data blocks, b. Component tuning, based on which 2 was selected as the
842 number of components to use; ER = error rate, BER = balanced error rate, error bars = standard
843 deviations, c. Plots of samples and sample classes per data type; green triangles with dashed lines
844 = controls, black circles with solid lines = PD and iRBD patients, ellipses represent 95%
845 confidence d. Correlation circle plot showing the selected features together, with labels for MG
846 and MT taxa and metabolites, e. Loadings for the five selected features for each data type and
847 component. Annotations for the selected KOs are provided in Supplementary Table 10.

848 **Extended Data Figure 6. Additional results on 2-hydroxypyridine and genus**
849 ***Methanobrevibacter*.** a. \log_{10} (relative abundance) of metagenomic reads classified as
850 *Methanobrevibacter smithii* by diagnosis group in main study cohort, b. \log_{10} (relative abundance)
851 of metatranscriptomic reads classified as *Methanobrevibacter smithii* by diagnosis group in main

852 study cohort, c. % of metaproteomic spectra classified as *Methanobrevibacter* by diagnosis group
853 in the main study cohort, d. log₁₀(relative abundance) of 16S rRNA gene amplicons classified as
854 *Methanobrevibacter* in the Luxembourgish NCER- PD cohort. e. Summaries of *q*-values (fdr-
855 adjusted *P*-values) of correlations for 2-hydroxypyridine and 74 methanogenesis-related genes
856 found in the sequence data (tested either in full data or with data containing samples of a specific
857 group, as given on the x-axis). f. % of samples with predicted proteins containing the HmdB or
858 HmdC genes, which code for the biosynthesis of the cofactor for Hmd. g 2-hydroxypyridine
859 normalized peak area depending on the presence of HmdB or HmdC gene in samples. MG =
860 metagenomic data, MT = metatranscriptomic data; in boxplots, box hinges: 1st and 3rd quartiles;
861 whiskers: hinge to highest/lowest values that is within 1.5*IQR of hinge; grey cross: mean.

862 **Extended Data Figure 7. Additional results from the HiTox yeast model.** a. Representative
863 growth curves of control and HiTox strains in presence of 2-hydroxypyridine (2-HP) at the
864 indicated concentrations. b. 2-HP dose-response assay in uninduced condition in HiTox and
865 control strains. c. Dose-response assay in control strain and HiTox strain in aSyn-expressing
866 condition after treatment with either 2-HP, 3-hydroxypyridine (3-HP) or 4- hydroxypyridine (4-
867 HP). In b and c, OD₆₀₀ was measured 48 h after inoculation and mean and SD were calculated
868 from four biological replicates.

869 **Extended Data Figure 8. Additional results from the induced human pluripotent stem cell**
870 **enteric neuron model.** a. Induced human pluripotent stem cell characterization stainings show
871 robust expression of OCT4, SEEA4 and NANOG pluripotency markers. Scale bars: 200 μm. b.
872 Enteric neuron characterisation stainings at 40 days of differentiation detect presence of neural
873 crest stem cell markers MASH1 and PHOX2A, and of different neuronal identities, such as
874 GABAergic, serotonergic and dopaminergic. Scale bars: 100 μm. c. Relative viability after 2-

875 hydroxypyridine exposure. Comparison to untreated sample was not significant when evaluated
876 with a Kruskal-Wallis test. d. High-content imaging of apoptosis marker cleaved-caspase 3 (CC3),
877 TUJ1-positive neurons and Hoechst-positive nuclei. Nuclei images contain a representation of the
878 mask applied to segment the nuclei; scale bar: 100 μm . e. Quantification of CC3-positive staining
879 normalized to the amount of TUJ1-positive neurons. In c and d, data is represented as mean \pm SEM
880 of three independent neuronal differentiations, with 30 fields per well and 8 wells per condition
881 quantified for each. ** $P < 0.01$.

882 **Extended Data Figure 9. Effects of intrastriatal injections of 100 mM 2-hydroxypyridine in**
883 **transgenic mouse model of PD on phospho-aSyn accumulation in different brain regions.** a.
884 Striatum: 2-HP-induced decrease of phospho-aSyn signals in synaptic boutons; scale bar: 50 μm ,
885 b. Substantia nigra: 2-HP-induced non-significant increase of phospho-aSyn in synaptic boutons;
886 scale bar: 350 μm , c. Prefrontal cortex: 2-HP-induced significant increase of phospho-aSyn signals
887 in cell body profiles with neuronal morphology (double-stained with DAPI, lower row of images);
888 scale bar: 50 μm . Microphotographs show examples for 0 mM and 100 mM in hSNCA mice only.
889 Bar plots show mean and standard deviation; n of mice = 5-8 per group. P -values are given between
890 group means that are different. * $P < 0.05$.

891

Supplementary Files

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