Title

Prolonged nicotine exposure reduces aversion to the drug in mice by altering nicotinic transmission in the interpeduncular nucleus.

Authors

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Abstract

Nicotine intake is likely to result from a balance between the rewarding and aversive properties of the drug, yet the individual differences in neural activity that control aversion to nicotine and their adaptation during the addiction process remain largely unknown. Using a two-bottle choice experiment, we observed a high heterogeneity in nicotine-drinking profiles in isogenic adult male mice, with about half of the mice persisting in consuming nicotine even at high concentrations, whereas the other half stopped consuming. We found that nicotine intake was negatively correlated with nicotine-evoked currents in the interpeduncular nucleus (IPN), and that prolonged exposure to nicotine, by weakening this response, decreased aversion to the drug, and hence boosted consumption. Lastly, using knock-out mice and local gene re-expression, we identified β4-containing nicotinic acetylcholine receptors of IPN neurons as the molecular and cellular correlates of nicotine aversion. Collectively, our results identify the IPN as a substrate of individual variabilities and adaptations in nicotine consumption.

42 **Introduction**

43

44 Nicotine remains one of the most-widely used addictive substance in the world, and
45 even though cigarette smoking is overall decreasing, the use of new products such as
46 electronic cigarettes has risen dramatically in recent years [1]. Nicotine administration
47 induces a series of effects, ranging from pleasant (i.e. appetitive, rewarding,
48 reinforcing, anxiolytic…) to noxious (i.e. anxiogenic, aversive…) [2,3]. These multifaceted
49 effects have been described both in humans and in rodents. They greatly depend on
50 the dose of nicotine administered, show substantial inter-individual variability, and are
51 believed to be key in the regulation of nicotine intake and in the maintenance of
52 addiction [2-4]. Understanding the variable effects of nicotine at the molecular and circuit
53 levels is therefore fundamental to progress in the pathophysiology of nicotine addiction,
54 and to develop efficient smoking-cessation therapies.

55

56 Nicotine mediates its physiological effects by activating nicotinic acetylcholine
57 receptors (nAChRs), pentameric ligand-gated ion channels encoded by a large
58 multigene family [2]. There are nine nAChR α (α2-10) and three β (β2-4) subunits
59 expressed in the brain, which can assemble to form homo-pentamers or hetero-
60 pentamers with various localizations and functions [5,6]. Initiation of consumption and
61 reinforcement to nicotine involve the mesolimbic dopamine reward circuit, which
62 originates in the ventral tegmental area (VTA) [7]. Nicotine primarily acts on this circuit
63 by activating α4β2 nAChRs, a receptor subtype that displays high affinity for the drug
64 [7-10]. Interestingly, an acute injection of nicotine also inhibits a subset of VTA dopamine
65 neurons that project to the amygdala [11], and this results in elevated anxiety in mice,

66 illustrating the heterogeneity of the brain reward circuit, and the complexity of nicotine
67 dependence.
68
69 Another important pathway in the neurobiology of nicotine addiction is the medial
70 habenulo-interpeduncular (MHb-IPN) axis [12-15]. This pathway is deeply implicated in
71 the regulation of aversive physiological states such as fear and anxiety [16-18]. It is
72 believed to directly mediate aversion to high doses of nicotine [12,13], to trigger the
73 affective (anxiety) and somatic symptoms following nicotine withdrawal [19-22] and to be
74 involved in relapse to nicotine-seeking [23]. Strikingly, neurons of the MHb-IPN axis
75 express the highest density and largest diversity of nAChRs in the brain, notably the
76 rare α5, α3 and β4 subunits [6]. These are encoded by the CHRNA5-A3-B4 gene cluster,
77 of which some sequence variants are associated with a high risk of addiction in humans
78 [24,25]. The α3 and β4 subunits are virtually absent in the VTA, or in other parts of the
79 brain. The α3β4 nAChR shows lower affinity for nicotine than the α4β2 subtype, which
80 has contributed to the widely accepted idea that nicotine is rewarding at low doses
81 because it activates primarily α4β2 receptors of the VTA, while it is aversive at high
82 doses because only then does it activate α3β4 nAChRs of the MHb-IPN axis [12,14].
83 Nevertheless, this hypothesis of different sensitivity to nicotine in different circuits is
84 based on indirect evidence from c-fos quantification or brain slice physiology
85 experiments, and does not take into account the possible adaptive changes in the
86 circuits that result from repeated exposure to nicotine. Therefore, recording the
87 physiological response of IPN neurons to nicotine *in vivo*, both in naive animals and
88 after prolonged exposure to nicotine, remains a prerequisite to understand the
89 mechanism by which smokers develop tolerance to the aversive effects of nicotine.
90

91 A distinct feature of addiction is that, overall, only some individuals lose control over
92 their drug use, progressively shifting to compulsive drug intake [26-30]. About a third to
93 half of those who have tried smoking tobacco become regular users [31]. Individual
94 differences in the sensitivities of the VTA and MHb-IPN systems, and in their respective
95 adaptations during chronic tobacco use, could contribute to the vulnerability to nicotine
96 and to the severity of the addiction process [14,32-35]. Yet, the neural mechanism that
97 makes individuals more prone to maintain nicotine consumption versus durably stop
98 are unclear. In addition, whether the MHb-IPN pathway respond differently to nicotine
99 in individuals with and without a history of nicotine use is largely unknown. Here we
100 used isogenic mice and electrophysiology (*ex vivo* and *in vivo*) to study the neuronal
101 correlates of inter-individual variabilities in nicotine consumption behavior, and their
102 adaptation after chronic exposure to the drug.
103
104
105 **Results**
106
107 <u>Heterogeneity in nicotine consumption in isogenic wild-type mice.</u>
108
109 We used a continuous access, two-bottle choice nicotine-drinking test to assess
110 consumption profiles in wild-type (WT) C57Bl6 male mice single-housed in their home
111 cage (Fig. 1A). In this test, animals have continuous and concurrent access to two
112 bottles containing a solution of either 2% saccharine (vehicle) or nicotine plus 2%
113 saccharine (to mask the bitter taste of nicotine). After a 4-day habituation period with
114 water in both bottles, nicotine concentration was progressively increased in one bottle
115 across 16 days, from 10 to 200 μg/ml (4 days at each concentration), while alternating

116 the side of the nicotine-containing solution every other day to control for side bias (Fig.
117 1B). Consumption from each bottle was measured every minute. We found that the
118 daily nicotine intake increased throughout the paradigm, to stabilize at about 10
119 mg/kg/day on average for the highest nicotine concentration tested (Fig. 1C). Overall,
120 the percent of nicotine consumption, i.e., the nicotine solution intake relative to the total
121 fluid intake, was initially close to 50%, and decreased below that value for nicotine
122 concentrations above 50 μg/ml (Fig. 1D). These results match what was observed in
123 previous studies using C57Bl6 male mice, notably the fact that these mice rarely show
124 over 50% nicotine consumption, whether the water is supplemented with saccharine
125 or not [36,37]. The decreased percent nicotine consumption observed at the population
126 level in the course of the task suggests that mice adapt their behavior to reduce their
127 number of visits to the nicotine-containing bottle. We indeed observed that mice rapidly
128 (within a day) adjusted their nicotine intake when the concentration of nicotine
129 increases (Fig. S1A), resulting in titration of the nicotine dose, as previously reported
130 [12,34].
131
132 We noticed some disparity between mice and decided to analyze more precisely
133 nicotine consumption profiles for each individual. In particular, some mice abruptly
134 reduced their intake right after an increase in nicotine concentration in the bottle, or
135 had very low nicotine intake throughout the entire task (intake never exceeded 2
136 mg/kg/day) (Fig. 1E). We classified these mice (17/35) as “avoiders”. The other half of
137 the mice (18/35), in contrast, displayed a continuous increase in nicotine intake, or
138 eventually reached a titration plateau in their consumption. These mice were classified
139 as “non-avoiders”. Another way of looking at these distinct consumption profiles is to
140 quantify the differences in intake between two consecutive days (positive differences

141 indicate increased intake, while negative differences indicate decreased intake). The
142 distinction between the two phenotypic groups appears clearly when we plot, for each
143 mouse, the minimum and maximum values of the difference in intake between two
144 consecutive days (Fig. 1E, right). The group of avoiders was characterized either by a
145 minimum difference in intake that was negative (mice that reduce their intake abruptly),
146 or by both a minimum and a maximum difference in intake that were close to zero (very
147 low intake throughout the task). In contrast, the non-avoider mice were characterized
148 by a minimum difference in intake between two consecutive days that was always
149 positive, indicating continual increase in intake throughout the task. Overall, only a
150 small proportion of the mice (7/35, all non-avoiders) reached a plateau in their
151 consumption, which somewhat contrasts with the apparent titration observed at the
152 population level, either here (Fig. 1C) or in previous studies [12,34]. Avoiders and non-
153 avoiders (phenotypes defined as above throughout the manuscript) showed on
154 average similar nicotine intake for low concentrations of nicotine (10 and 50 μg/ml, $p >$
155 0.05), but while nicotine intake increased throughout the task for non-avoiders (16.9 ±
156 2.9 mg/kg/day for 200 μg/ml of nicotine) it dropped down to 2.6 ± 0.4 mg/kg/day for
157 such high nicotine concentration in avoiders (Fig. 1F), and almost reached zero over
158 the last three days (Fig. 1G). The percent nicotine consumption was fairly constant
159 throughout the task in non-avoiders, whereas in avoiders, it drastically decreased as
160 nicotine concentration increased, to approximate zero at the end of the task (Fig. S1B).
161 We then compared the level of aversion produced by nicotine in avoiders and non-
162 avoiders, with that produced by quinine, a notoriously bitter molecule. We found that
163 all of the naive mice actively avoided the quinine-containing solution, and showed a
164 percent quinine consumption close to zero, as observed with nicotine for avoiders, but

165 not for non-avoiders (Fig. S1C). Together, these results suggest that avoiders display
166 strong aversion to nicotine.
167
168 The concentration of nicotine triggering aversion differs between mice
169
170 Owing to the oral nature of the test, it may be difficult for mice to associate consumption
171 in a particular bottle with the physiological effects (whether positive or negative) of
172 nicotine. We thus systematically analyzed the percent nicotine consumption
173 throughout the task in individual mice, to examine their variability in choice patterns
174 and behavioral alterations. We found that some non-avoiders actively tracked the side
175 associated with nicotine when bottles were swapped (e.g. mouse #1 in Fig. 2A),
176 indicating strong preference for the bottle containing nicotine and active consumption.
177 Other non-avoiders displayed a strong side preference and never alternated drinking
178 side (e.g. mouse #2 in Fig. 2A), and hence consumed nicotine in a more passive
179 fashion. In contrast, all avoider mice (n = 17) displayed avoidance of the nicotine-
180 containing solution, whether they initially tracked the nicotine solution (e.g. mice #3
181 and 4 in Fig. 2A) or not (e.g. mice #5 and 6 in Fig. 2A). To quantify the evolution of
182 nicotine consumption at the individual level throughout the task, and to better take into
183 account the passive consumption behavior of some of the mice, we mapped each
184 profile in a pseudo-ternary plot where the base represents the nicotine consumption
185 index (from 0 to 100%), while the top apex represents 100% side bias (Fig. 2B). Such
186 a ternary representation enables us to graphically distinguish between mice that
187 actively track the nicotine bottle (bottom right apex, 100% nicotine consumption index),
188 mice that actively avoid nicotine (bottom left apex, 0% nicotine consumption index),
189 and mice that have a strong side bias (top apex), and to calculate the shortest distance

190 for each mouse to each of the three apices. In addition, by representing the trajectory
191 for each individual from the water condition to the 200 μg/ml nicotine condition, this
192 plot can be used to reveal and quantify behavioral adaptations (or lack thereof) in each
193 individual. Overall, we found that the behavior of non-avoiders was on average fairly
194 consistent throughout the task, i.e. their distance from the three apices was not really
195 impacted by the modifications in nicotine concentration (Fig. 2C and Fig. S2), whether
196 they consumed nicotine actively or passively. In contrast, the behavior of avoiders was
197 highly nicotine concentration-dependent, with mice going further away from the 100%
198 nicotine apex as nicotine concentration increases (Fig. 2C and Fig. S2A-B). Aversion
199 to nicotine in avoider mice mainly occurred at the transition from 100 to 200 μg/ml, but
200 some mice displayed aversion at concentrations as low as 10 μg/ml (Fig. 1E, 2A and
201 2C). Together, these results show that most mice can actively track or avoid nicotine,
202 indicating that they can discriminate nicotine from the control solution. Avoiders started
203 actively avoiding the nicotine-containing bottle once a specific drug concentration was
204 reached, suggesting the existence of a threshold at which nicotine aversion is
205 triggered, which apparently differs between mice.
206
207 Do avoiders learn to keep away from the nicotine-containing solution, or do they just
208 rapidly react to the nicotine concentration in the bottle to adjust their daily intake? To
209 answer this question, we added at the end of the two-bottle choice task, i.e. after the
210 200 μg/ml nicotine concentration, a condition with a low concentration of nicotine (50
211 μg/ml) for four days. We chose 50 μg/ml of nicotine because avoiders and non-
212 avoiders initially displayed comparable nicotine intake (Fig. 1F) and percent
213 consumption (Fig. S1B) at this concentration. We hypothesized that if avoiders
214 increased their percent nicotine consumption at the 200 to 50 μg/ml transition, it would

215 indicate a rapid adjustment to the concentration proposed, so to maintain their level of
216 intake constant. In contrast, if avoiders maintained a steady, low percent nicotine
217 consumption, it would indicate that nicotine aversion persists, independently of the
218 dose. We indeed found that lowering nicotine concentration from 200 to 50 µg/ml did
219 not increase percent nicotine consumption in avoiders (Fig. 2D), at least for the four
220 days mice were subjected to this concentration, suggesting aversion learning for
221 nicotine, and a behavioral adaptation that leads to near complete cessation of nicotine
222 consumption.
223
224 Nicotine consumption negatively correlated with the amplitude of nicotine-evoked
225 currents in the IPN
226
227 We then investigated the neural correlates of such aversion to nicotine. We
228 hypothesized that the IPN, which is involved in nicotine aversion and in negative
229 affective states [3,38,39], might be differently activated by nicotine in avoiders and non-
230 avoiders. Therefore, we used whole-cell patch-clamp recordings in brain slices to
231 assess, at completion of the two-bottle choice task, the functional expression level of
232 nAChRs in IPN neurons. We recorded neurons from the dorsal and rostral IPN
233 because these neurons have high nAChR density [14,15,40,41]. To record nicotine-evoked
234 currents, we used a local puff application of nicotine at a concentration (30 µM) close
235 to the EC50 for heteromeric nAChRs [42]. We found that the amplitude of nicotine-
236 evoked currents was higher in IPN neurons of avoider mice than in non-avoiders (Fig.
237 2E). Moreover, we found a negative correlation between the average amplitude of
238 nicotine-evoked current in IPN neurons, and nicotine consumption (measured over the
239 last 24 hours prior to the patch-clamp recording, Fig. 2F), suggesting that nicotine

240 consumption in mice is negatively linked to the amplitude of the response to nicotine
241 in IPN neurons.
242
243 Chronic nicotine treatment alters both nicotinic signaling in the IPN and nicotine
244 consumption.
245
246 It is still unclear at this stage whether chronic nicotine exposure progressively alters
247 the response of IPN neurons to the drug (non-avoiders being further exposed to high
248 nicotine doses than avoiders) or whether an intrinsic difference pre-exists in avoiders
249 and non-avoiders. To determine the effect of chronic nicotine exposure on nAChR
250 current levels in IPN neurons, we passively and continuously exposed mice to nicotine
251 for 4 weeks, using subcutaneously implanted osmotic minipumps. The concentration
252 of nicotine in the minipump (10 mg/kg/day) was chosen to match the average voluntary
253 nicotine intake in the two-bottle choice task (Fig. 1C). We then recorded from acute
254 brain slices and found that indeed, prolonged exposure to nicotine reduced the
255 amplitude of nicotine-evoked currents in the IPN of these mice compared to control
256 mice treated with saline (Fig. 3A). These results are consistent with the reduced current
257 amplitudes observed in mice that underwent the two-bottle choice task, compared to
258 naive mice in their home cage (Fig S3A). Because IPN neurons are mostly silent in
259 brain slices, and in order to preserve the entire circuitry intact, we decided to perform
260 juxtacellular recordings of IPN neurons *in vivo*, and to characterize their response
261 (expressed in % of variation from baseline) to an intravenous (i.v.) injection of nicotine
262 (30 µg/kg). To our knowledge, no description of *in vivo* recordings of IPN neurons, and
263 thus no criteria for identification, have been reported as yet, hence we solely
264 considered neurons that were labelled *in vivo* with neurobiotin and confirmed to be

265 within the IPN for the analysis. We found that nicotine i.v. injections in naive WT mice
266 induced an increase in the firing rate of IPN neurons compared to an injection of saline,
267 and that this acute effect of nicotine was reduced after the prolonged (4 weeks) passive
268 exposure of mice to the drug (Fig.3B). Some of the IPN neurons, however, responded
269 to nicotine by decreasing their firing rate, and the degree of inhibition was lower in the
270 group exposed to chronic nicotine (Fig. S3B). Together, these *ex vivo* and *in vivo*
271 recordings demonstrate that prolonged exposure to nicotine markedly reduces
272 nicotine-evoked responses in mouse IPN neurons.
273
274 To verify the hypothesis that a modification in the cholinergic activity of IPN neurons
275 impacts nicotine aversion, we did a series of experiments. Firstly, we evaluated the
276 consequence of prolonged nicotine exposure on nicotine consumption. Mice were
277 implanted with an osmotic minipump to passively deliver nicotine and, after 20 days,
278 were subjected to a modified two-bottle task that consisted in a rapid presentation to a
279 high concentration (100 µg/ml) of nicotine (Fig. 3C). We chose this protocol to avoid
280 the confounding effects of a gradual exposure to nicotine, and to evoke strong aversion
281 in mice. We found that indeed, mice pretreated with saline (controls) showed low
282 percent nicotine consumption (Fig. 3C), which may indicate aversion to such high
283 nicotine concentration. In contrast, mice pretreated with nicotine did not decrease on
284 average their percent consumption when nicotine was introduced in the bottle,
285 suggesting that they may have developed tolerance for the aversive effects of nicotine.
286 When looking at the data day by day, we observed that control mice abruptly
287 decreased their percent consumption when nicotine was introduced, while for nicotine-
288 treated animals the decrease was more gradual over the four days (Fig. S3C). Overall,
289 this resulted in greater nicotine intake for the group treated with nicotine than for the

290 group treated with saline (Fig. 3C). When focusing on individuals, we observed that a
291 single saline-pretreated mouse (1/23) increased its percent consumption when
292 nicotine was introduced in the task, while the great majority of the mice actively avoided
293 nicotine (close to the 0% Nicotine apex). In contrast, a marked proportion of the mice
294 treated with nicotine (8/25) increased their percent consumption when nicotine was
295 introduced (Fig. 3D, p-value = 0.037 Chi-squared). The two groups were identical in
296 the water/water session (Fig. 3E, top). However, in the water/nicotine session, mice
297 pretreated with nicotine showed a greater distance from the 0% nicotine consumption
298 apex, and a shorter distance from the 100% nicotine consumption apex than mice
299 pretreated with saline (Fig. 3E, bottom), indicating decreased aversion and increased
300 preference for nicotine in the nicotine-pretreated group. Altogether, these
301 electrophysiological and behavioral data demonstrate that prolonged exposure to
302 nicotine both decreases nicotine efficacy in the IPN, and also decreases aversion to
303 nicotine in individuals, resulting in increased consumption of the drug. Yet, chronic
304 nicotine can produce adaptations in other brain circuits, and whether the
305 neurophysiological changes observed in the IPN are causally related to the variations
306 in aversion sensitivity remains to be demonstrated.
307
308 Nicotine avoidance involves β4*nAChRs.
309
310 We turned to mutant mice deleted for the gene encoding the nAChR β4 subunit ($\beta4^{-/-}$
311 mice), because of the strong and restricted expression of this subunit in the MHb-IPN
312 pathway [32,43,44]. We found that $\beta4^{-/-}$ mice displayed both greater percent nicotine
313 consumption and greater nicotine intake than WT animals, with minimal concentration-
314 dependent change in percent nicotine consumption (Fig. 4A). When looking at

315 individuals, we observed both active and passive nicotine-drinking profiles in β4$^{-/-}$ mice,
316 as already observed in WT mice. Strikingly however, none of the β4$^{-/-}$ mice (0/13)
317 showed aversion-like behavior at high nicotine concentration, which contrasts with the
318 high proportion of avoiders in WT animals (17/35, Fig. 4B, Fig. S4A, p =0.04 Pearson's
319 Chi squared with Yates 'continuity correction). WT mice showed a strong nicotine
320 concentration-dependent adaptation in their behavior, while β4$^{-/-}$ mice had a more
321 consistent behavior throughout the task (Fig. 4C and Fig. S4B, C).
322
323 To verify that responses to nicotine were affected in IPN neurons of β4$^{-/-}$ mice, we
324 performed whole-cell patch-clamp recordings. We found that the amplitude of nicotine-
325 evoked currents in the IPN was on average three-fold lower in β4$^{-/-}$ than in WT mice
326 (Fig. 4D), confirming that β4*nAChRs are the major receptor subtype in the IPN.
327 Furthermore, prolonged nicotine treatment had no significant effect on the amplitude
328 of nAChR currents in these knock-out mice (Fig. 4D), suggesting that the
329 downregulation observed after chronic nicotine treatment in the IPN of WT mice mainly
330 affects β4*nAChRs. We then used *in vivo* juxtacellular recordings, and performed
331 dose-response experiments (7.5 - 30 μg/kg) in order to assess the role of β4*nAChRs
332 in the response to different doses of nicotine. In WT mice, nicotine i.v. injections
333 resulted in a dose-dependent increase in IPN neuron activity (Fig. 4E). In β4$^{-/-}$ mice,
334 responses to nicotine were of smaller amplitude, especially for the highest dose of
335 nicotine tested (Fig. 4E), further demonstrating the important role of β4*nAChRs in the
336 response of the IPN to nicotine. In both WT and β4$^{-/-}$ mice, we observed a population
337 of neurons that decreased their firing rate in a dose-dependent manner, yet with no
338 difference in the amplitude of the response between the two genotypes (Fig. S4D-F),
339 suggesting that β4*nAChR are mainly involved in the increase, but not in the decrease,

340 of neuronal activity in response to nicotine injection. Collectively, our results in $\beta4^{-/-}$
341 mice demonstrate the key role of the nAChR β4 subunit in signaling aversion to
342 nicotine, and its predominant function in the activation of the IPN by nicotine.
343
344 <u>β4*nAChRs of the IPN are critically involved in nicotine aversion.</u>
345
346 β4 nAChRs are enriched in the IPN, yet they are also expressed to some extent in
347 other brain regions. Hence, to directly implicate β4*nAChRs of IPN neurons in nicotine
348 aversion, and more generally in nicotine consumption, we targeted re-expression of β4
349 in the IPN specifically, using lentiviral vectors in $\beta4^{-/-}$ mice (KO-$\beta4^{IPN}$ mice, Fig. 5A).
350 Mice transduced with eGFP (KO-GFP^{IPN} mice) were used as controls. Proper
351 transduction in the IPN was verified using immunohistochemistry after completion of
352 the two-bottle choice task (Fig. 5A), and mice with expression of GFP in the VTA were
353 excluded from analyses. Transduction of β4, but not of GFP alone, in the IPN increased
354 the amplitude of nicotine-evoked currents (Fig. 5B) and restored levels found in WT
355 animals (U test, $p = 0.6$, Fig. S5A). We compared nicotine intake in the two groups of
356 mice in the two-bottle choice task. We found that re-expression of β4 in the IPN of $\beta4^{-}$
357 $^{/-}$ mice decreased nicotine intake compared to the group of mice transduced with eGFP
358 in the IPN (Fig. 5C). Overall, nicotine intake was similar in WT and in KO-$\beta4^{IPN}$ animals
359 ($p>0.5$ for all concentrations), demonstrating the causal role of β4 nAChRs of IPN
360 neurons in nicotine consumption behaviors. At the individual level, the proportion mice
361 that avoided nicotine at 200 μg/kg was very low for KO-GFP^{IPN} control mice (2/18), but
362 greater for KO-$\beta4^{IPN}$ mice (8/17, Fig. 5D, $p = 0.04$ Chi squared). The behavior of KO-
363 GFP^{IPN} was steady throughout the task, whereas it was highly nicotine concentration-
364 dependent for KO-$\beta4^{IPN}$ mice (Fig. 5E and Fig. S5B,C), as already observed with WT

365 mice. Mice with strong percent nicotine consumption were only found in the KO-GFP^{IPN}
366 control group. Collectively, these results show that selective re-expression of β4 in the
367 IPN of β4$^{-/-}$ mice rescued aversion for nicotine, and highlight the specific role of
368 β4*nAChRs of IPN neurons in signaling aversion to nicotine, and in the control of
369 nicotine intake.
370
371 **Discussion**
372
373 We used a two-bottle choice paradigm to assess inter-individual differences in nicotine
374 consumption in mice, and to evaluate how pre-exposure to nicotine modifies drug
375 taking. Oral self-administration is a classical method for chronic nicotine administration
376 as it provides rodents with *ad libitum* access to nicotine, likely mimicking administration
377 in human smokers, while minimizing stress from handling [45]. We observed that, on
378 average, WT mice titrate their intake to achieve a consistent nicotine dose, in
379 agreement with previous reports [12,34,35], and discovered that mutant mice lacking the
380 nAChR β4 subunit did not, resulting in greater nicotine intake, notably at high nicotine
381 concentrations in the drinking solution. These behavioral results are in agreement with
382 the greater intracranial self-administration observed at high nicotine doses in these
383 mice [33] and, conversely, with the results obtained with transgenic TABAC mice
384 overexpressing the β4 subunit at endogenous sites, which avoid nicotine and
385 consequently consume very little [13,33]. Nevertheless, it should be noted that conflicting
386 results have also been reported regarding the role of β4 nAChRs in nicotine
387 consumption. Notably, intravenous self-administration of nicotine is lower in β4$^{-/-}$ mice
388 despite a higher sensitivity of the VTA to nicotine in these mice [32], and self-
389 administration is higher in TABAC mice despite reduced nicotine-induced activation of

390 the VTA [46]. The increased consumption at high nicotine concentration reported here
391 for $\beta4^{-/-}$ mice resembles what was observed in mutant mice either lacking the $\alpha5$
392 subunit [12] or with low levels of the $\alpha3$ subunit [47], likely because the $\alpha3$, $\alpha5$, and $\beta4$
393 nAChR subunits, which are encoded by the same gene cluster, co-assemble in brain
394 structures, notably the MHb-IPN pathway, to produce functional heteromeric nAChRs
395 that contribute to the control of nicotine intake.
396
397 In the two-bottle choice nicotine-drinking test, a percent nicotine consumption below
398 50% is usually interpreted as a sign of aversion to the drug [13,37]. However, it may rather
399 indicate a process of regulation of nicotine consumption, through which mice adapt to
400 an increase in nicotine concentration by reducing the volume of nicotine intake at each
401 visit. At low nicotine concentrations, percent nicotine consumption never really
402 exceeded 50% in average, as already seen in previous studies [37], yet very few mice
403 readily avoided the nicotine-containing bottle, and many mice did track nicotine, which
404 would rather indicate an actual appetite (as opposed to an aversion) for the drug. As
405 nicotine concentration increases, the percent nicotine consumption dropped to about
406 20% on average at the end of the task (Fig. 1D), yet it was associated with an increase
407 in nicotine intake (in mg/kg/day, Fig. 1C). Hence the nicotine preference score alone
408 may not be sufficient to determine whether nicotine is aversive or not. We believe that,
409 instead, aversion to the drug may be better defined by a constantly low consumption,
410 or a sudden drop in nicotine intake, as seen here with the group of avoider mice (Fig.
411 1E-G).
412
413 One important limitation of population-level analyses is that they greatly limit the ability
414 to examine inter-individual differences in drug taking behaviors. It is indeed

415 increasingly acknowledged that in mice, as in humans, there is a substantial variability
416 in the susceptibility for developing drug use disorders [27-29,48-51]. Yet, it is still unclear
417 why some individuals are more susceptible than others to become regular users. Here
418 we inspected drinking profiles in individual isogenic mice, and discovered large inter-
419 individual differences in nicotine vulnerability: about half of the WT mice, the avoiders,
420 durably quit nicotine at a certain concentration, whereas the other half, the non-
421 avoiders, continued consumption even at high concentration of nicotine, classically
422 described as aversive [3]. Avoiders displayed variable concentration thresholds required
423 for triggering aversion, and some of them even developed aversion at the beginning of
424 the two-bottle choice task, when concentrations of nicotine were still low. This finding,
425 which challenges the popular idea that aversion is only triggered by high doses of
426 nicotine [3,12-14], is somewhat supported by our electrophysiogical data. Indeed, by
427 directly measuring the response of IPN neurons to different doses of nicotine *in vivo*,
428 we show that concentrations of nicotine as low as 7.5 μg/kg can engage the IPN
429 circuitry, suggesting that nicotine-induced signaling can emerge in this structure even
430 at low drug concentration. Non-avoiders (WT and $\beta4^{-/-}$ mice) may retain an aversion
431 threshold, but which would exceed the highest concentration tested here. Importantly,
432 very few mice showed what could be considered as titration (plateaued consumption),
433 emphasizing the needs to consider individual behavior, as opposed to group behavior,
434 in addiction research.
435
436 We also discovered that the functional expression level of β4-containing nAChRs in
437 the IPN underlies these different sensitivities to the aversive properties of nicotine.
438 Indeed, inter-individual variability for nicotine aversion was nearly eliminated in $\beta4^{-/-}$
439 mice, none of which quit drinking nicotine, and was restored after selective re-

440 expression of the β4 subunit in the IPN. This selective re-expression experiment
441 discards the possibility that different sensitivities to the bitter taste of nicotine solutions
442 explain the different drinking profiles of avoiders and non-avoiders. Strikingly, we
443 observed a negative correlation between nicotine consumption and the response of
444 the IPN to the drug: mice consuming large amounts of nicotine displayed lower
445 nicotine-evoked currents in the IPN than mice consuming small amounts of nicotine,
446 which displayed large currents. The expression level of β4-containing nAChRs in the
447 IPN may thus determine the level of aversion to the drug, and consequently its intake.
448 We suggest that β4-containing nAChRs, by engaging the IPN circuitry, initiate a
449 primary response to nicotine that, if above a certain threshold, will trigger acute
450 aversion to the drug, thus impacting the balance between drug reward and aversion to
451 limit drug consumption. In line with this, it was found that pharmacological or
452 optogenetic stimulation of the MHb-IPN pathway could directly produce aversion
453 [14,15,34], while pharmacological inactivation of this pathway increases nicotine intake [12].
454 From our results, we further suggest that the aversion produced by nicotine is not just
455 an acute response to the dose that has just been ingested. Aversion can also be lasting
456 for days, as evidenced by the fact that mice persist in avoiding the nicotine-containing
457 bottle even after the concentration has been reduced to a dose they used to ingest.
458 This echoes observations made in humans, in which the unpleasant initial responses
459 to cigarettes is associated with a reduced likelihood of continued smoking [52]. Such a
460 sustained aversive reaction to nicotine was conditioned, in mice, by nicotine itself, and
461 required β4-containing nAChRs of the IPN for its onset, but most likely involves other
462 brain circuits for its persistence in the long-term. Identifying the molecular and cellular
463 mechanism of long-term aversion to nicotine in mice will be instrumental to progress
464 in our understanding of human dependence to tobacco.

465

466 We did not observe major differences in nicotine intake between avoiders and non-
467 avoiders at the beginning of the two-bottle choice experiment, for low concentrations
468 of nicotine (< 100 μg/ml), but cannot completely rule out pre-existing inter-individual
469 differences that may explain the opposite trajectories taken by the two groups. Indeed,
470 the mice used in this study were isogenic, yet epigenetic changes during development
471 or differences in social status, which are known to affect brain circuits and individual
472 traits [53], may affect the responses of the IPN to nicotine. It is indeed tempting to
473 speculate that external factors (e.g. stress, social interactions…) that would affect the
474 expression level of β4-containing nAChRs in the IPN, will have a strong impact on
475 nicotine consumption. In addition to pre-existing differences, history of nicotine use
476 could produce long-lasting molecular and cellular adaptations in the IPN circuitry that
477 may alter nicotine aversion and consumption. In the VTA, chronic nicotine upregulates
478 the number of β2-containing receptors at the cell surface [54,55]. We discovered that
479 prolonged nicotine exposure had the opposite effect on β4-containing nAChRs of the
480 IPN: it downregulated and/or desensitized these receptors, as evidenced by the
481 decreased response to nicotine both *ex vivo* and *in vivo*. This effect seems to be
482 specific to β4-containing nAChRs, since chronic nicotine had minimal consequence on
483 the residual IPN nAChR current in $\beta4^{-/-}$ mice. These results may appear at odds with
484 the recent report of increased nAChR currents in IPN slices of nicotine-treated mice [56].
485 However, differences in the effective agonist concentrations used in the two studies
486 (30 μM of nicotine with local puff here, *vs.* 50 μM of caged-nicotine, but effective
487 concentration probably much lower after photo-uncaging in [56]) may explain this
488 discrepancy. Indeed, by using a lower effective agonist concentration, Arvin et al. [56]
489 were possibly not recruiting the low-affinity β4-containing receptors, which are the

490 receptor subtypes we found to be downregulated by chronic nicotine exposure (Fig.
491 4D). Whatever the reason behind these opposite results is, our behavioral data in
492 nicotine-treated WT and $\beta4^{-/-}$ mice completely match: both displayed reduced
493 responses to nicotine (*ex vivo* and i*n vivo)* in IPN neurons, as well as increased nicotine
494 intake compared to naive, WT animals. We suggest that long-term exposure to nicotine
495 decreases the likelihood to reach the threshold at which mice develop aversion to the
496 drug, which ultimately leads to increased drug consumption. In other words, nicotine
497 intake history weakens the ability of nicotine to induce aversion in mice. In most
498 nicotine replacement therapies, such as gums or patches, nicotine is slowly
499 administered over prolonged periods of time, to supposedly attenuate the negative
500 emotional reactions elicited by nicotine withdrawal [57]. However, our data indicate that
501 mice under prolonged nicotine administration will also develop tolerance to the
502 aversive effects of nicotine, highlighting the necessity to develop alternative medical
503 approaches.

504 **Materials and Methods**

505 *Animals*

506 Eight to sixteen-week old wild-type C57BL/6J (Janvier labs, France) and Acnb4 knock-
507 out ($\beta4^{-/-}$) mice (Pasteur Institute, Paris) [58] were used for this study. $\beta4^{-/-}$ mice were
508 backcrossed onto C57BL/6J background for more than twenty generations. Mice were
509 maintained on a 12h light-dark cycle. All experiments were performed in accordance
510 with the recommendations for animal experiments issued by the European
511 Commission directives 219/1990, 220/1990 and 2010/63, and approved by Sorbonne
512 Université.

513

514 *Two-bottle choice experiment*

515 Mice single-housed in a home cage were presented with two bottles of water (Volvic)
516 for a habituation period of 4 days. After habituation, mice were presented with one
517 bottle of saccharine solution (2%, Sigma Aldrich) and one bottle of nicotine (free base,
518 Sigma Aldrich) plus saccharine (2%) solution diluted in water (adjusted to pH ~7.2 with
519 NaOH). Unless otherwise noted, four different concentrations of nicotine were tested
520 consecutively (10, 50, 100 and 200 µg/ml) with changes in concentration occurring
521 every 4 days. For the two-bottle aversion task, a single nicotine concentration (100
522 µg/ml) was used after the habituation period. Bottles were swapped every other day to
523 control for side preference. The drinking volume was measured every minute with an
524 automated acquisition system (TSE system, Germany). Nicotine intake was calculated
525 in mg of nicotine per kilogram of mouse body weight per day (mg/kg/d). To minimize
526 stress from handling, mice were weighed every other day, since we found their weigh
527 to be sufficiently stable over two days. Percent nicotine consumption was calculated
528 as the volume of nicotine solution consumed as a percentage of the total fluid

529 consumed. Mice showing a strong side bias (preference <20% or >80%) in the
530 habituation period were not taken into account for the analyses.
531 For the pseudo-ternary plot analyses, we determined the percent nicotine consumption
532 on the left-hand side (%c1) and the percent nicotine consumption on the right-hand
533 side (%c2), for each nicotine concentration and each animal. We then calculated the
534 nicotine consumption and side bias indexes, by plotting the minimum min(%c1, %c2)
535 against the maximum max(%c1, %c2), and used a 90° rotation to obtain the pseudo-
536 ternary plot. In this plot, the three apices represent mice that avoid nicotine on both
537 sides (0% nic.), mice that track nicotine on both sides (100% nic.), and finally mice that
538 drink solely one side (side biased).
539
540 *Prolonged treatment with nicotine*
541 Osmotic minipumps (2004, Alzet minipump) were implanted subcutaneously in 8-
542 week-old mice anesthetized with isoflurane (1%). Minipumps continuously delivered
543 nicotine (10 mg/kg/d) or saline (control) solution with a rate of 0.25 µl/h during 4 weeks.
544
545 *Brain slice preparation*
546 Mice were weighed and then anaesthetized with an intraperitoneal injection of a
547 mixture of ketamine (150 mg/kg, Imalgene 1000, Merial, Lyon, France) and xylazine
548 (60 mg/kg, Rompun 2%, Bayer France, Lyon, France). Blood was then fluidized by an
549 injection of an anticoagulant (0.1mL, heparin 1000 U/mL, Sigma) into the left ventricle,
550 and an intra-cardiac perfusion of ice-cold (0-4°C), oxygenated (95% O_2/5% CO_2)
551 sucrose-based artificial cerebrospinal fluid (SB-aCSF) was performed. The SB-aCSF
552 solution contained (in mM): 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4, 5.9 $MgCl_2$, 26 $NaHCO_3$,
553 25 sucrose, 2.5 glucose, 1 kynurenate (pH 7.2). After rapid brain sampling, slices (250

554 µm thick) were cut in SB-aCSF at 0-4°C using a Compresstome slicer (VF-200,
555 Precisionary Instruments Inc.). Slices were then transferred to the same solution at
556 35°C for 10 min, then moved and stored in an oxygenated aCSF solution at room
557 temperature. The aCSF solution contained in mM: 125 NaCl, 2.5 KCl, 1.25 NaH_2PO_4,
558 2 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 15 sucrose, 10 glucose (pH 7.2). After minimum 1h of
559 rest, slices were placed individually in a recording chamber at room temperature and
560 infused continuously with aCSF recording solution at a constant flow rate of about 2
561 ml/min.
562
563 *Ex vivo patch-clamp recordings of IPN neurons*
564 Patch pipettes (5-8 MΩ) were stretched from borosilicate glass capillaries (G150TF-3,
565 Warner instruments) using a pipette puller (Sutter Instruments, P-87, Novato, CA) and
566 filled with a few microliters of an intracellular solution adjusted to pH 7.2, containing (in
567 mM): 116 K-gluconate, 20 HEPES, 0.5 EGTA, 6 KCl, 2 NaCl, 4 ATP, 0.3 GTP and 2
568 mg/mL biocytin. Biocytin was used to label the recorded neurons. The slice of interest
569 was placed in the recording chamber and viewed using a white light source and a
570 upright microscope coupled to a Dodt contrast lens (Scientifica, Uckfield, UK). Neurons
571 were recorded from the dorsal (IPDL) and rostral (IPR) parts on the IPN. Whole-cell
572 configuration recordings of IPN neurons were performed using an amplifier (Axoclamp
573 200B, Molecular Devices, Sunnyvale, CA) connected to a digitizer (Digidata 1550
574 LowNoise acquisition system, Molecular Devices, Sunnyvale, CA). Signal acquisition
575 was performed at 10 kHz, filtered with a lowpass (Bessel, 2 kHz) and collected by the
576 acquisition software pClamp 10.5 (Molecular Devices, Sunnyvale, CA). Nicotine
577 tartrate (30 µM in aCSF) was locally and briefly applied (200 ms puffs) using a puff
578 pipette (glass pipette ~3 µm diameter at the tip) positioned about 20-30 µm from the

579 soma of the neuron. The pipette was connected to a Picospritzer (PV-800 PicoPump,
580 World Precision Instruments) controlled with pClamp to generate transient pressure in
581 the pipette (~2 psi). Nicotine-evoked currents were recorded in voltage-clamp mode at
582 a membrane potential of -60 mV. All electrophysiology traces were extracted and pre-
583 processed using Clampfit (Molecular Devices, Sunnyvale, CA) and analyzed with R.
584
585 *In vivo electrophysiology*
586 Mice were deeply anesthetized with chloral hydrate (8%, 400 mg/kg) and anesthesia
587 was maintained throughout the experiment with supplements. Catheters were
588 positioned in the saphenous veins of the mice to perform saline or nicotine intravenous
589 injections. Nicotine hydrogen tartrate salt (Sigma-Aldrich) was dissolved in 0.9% NaCl
590 solution and pH was adjusted to 7.4. The nicotine solution was injected at a dose of
591 7.5, 15 and 30 µg/kg. Borosilicate glass capillaries (1.5 mm O.D. / 1.17 mm I.D.,
592 Harvard Apparatus) were pulled using a vertical puller (Narishige). Glass pipettes were
593 broken under a microscope to obtain a ~1 µm diameter at the tip. Electrodes were filled
594 with a 0.5% NaCl solution containing 1.5% of neurobiotin tracer (AbCys) yielding
595 impedances of 6-9 MΩ. Electrical signals were amplified by a high-impedance amplifier
596 (Axon Instruments) and supervised through an audio monitor (A.M. Systems Inc.). The
597 signal was digitized, sampled at 25 kHz and recorded on a computer using Spike2
598 (Cambridge Electronic Design) for later analysis. IPN neurons were recorded in an
599 area corresponding to the following stereotaxic coordinates (4-5° angle): 3.3 - 3.6 mm
600 posterior to bregma, 0.2 - 0.45 mm from medial to lateral and 4.3 - 5 mm below the
601 brain surface. A 5 min-baseline was recorded prior to saline or nicotine i.v. injection.
602 For the dose-response experiments, successive randomized injections of nicotine (or

603 saline) were performed, interspaced with sufficient amount of time (> 10 min) to allow
604 the neuron to return to its baseline.
605
606 *Stereotaxic viral injections*
607 8-week old mice were injected in the IPN with a lentivirus that co-expresses the WT β4
608 subunit together with eGFP (or only eGFP for control experiments) under the control
609 of the PGK promoter. Lentiviruses were produced as previously described [7]. For viral
610 transduction, mice were anaesthetized with a gas mixture containing 1-3% isoflurane
611 (IsoVet®, Pyramal Healthcare Ltd., Nothumberland, UK) and placed in a stereotactic
612 apparatus (David Kopf Instruments, Tujunga, CA). Unilateral injections (0.1µl/min) of
613 1µl of a viral solution (Lenti.pGK.β4.IRES.eGFP, titer 150 ng/µl of p24 protein; or
614 Lenti.pGK.eGFP, titer 75 ng/µl of p24 protein) were performed using a cannula
615 (diameter 36G, Phymep, Paris, France). The cannula was connected to a 10 µL
616 Hamilton syringe (Model 1701, Hamilton Robotics, Bonaduz, Switzerland) placed in a
617 syringe pump (QSI, Stoelting Co, Chicago, IL, USA). Injections were performed in the
618 IPN at the following coordinates (5° angle): from bregma ML - 0.4 mm, AP - 3.5 mm,
619 and DV: - 4.7 mm (according to Paxinos & Franklin). Electrophysiological recordings
620 were made at least 4 weeks after viral injection, the time required for the expression of
621 the transgene, and proper expression was subsequently checked using
622 immunohistochemistry.
623
624 *Immunocytochemical identification*
625 Immunostaining was performed as described in [10], *with the following* primary
626 antibodies: anti-tyrosine hydroxylase 1:500 (anti-TH, Sigma, T1299) and chicken anti-
627 eYFP 1:500 (Life technologies Molecular Probes, A-6455). Briefly, serial 60 µm-thick

628 sections of the midbrain were cut with a vibratome. Slices were permeabilized for one
629 hour in a solution of phosphate-buffered saline (PBS) containing 3% bovine serum
630 albumin (BSA, Sigma; A4503). Sections were incubated with primary antibodies in a
631 solution of 1.5 % BSA and 0.2 % Triton X-100 overnight at 4°C, washed with PBS and
632 then incubated with the secondary antibodies for 1 hour. The secondary antibodies
633 were Cy3-conjugated anti-mouse (1:500 dilution) and alexa488-conjugated anti-
634 chicken (1:1000 dilution) (Jackson ImmunoResearch, 715-165-150 and 711-225-152,
635 respectively). For the juxtacellular immunostaining, the recorded neurons were
636 identified with the addition of AMCA-conjugated streptavidin (1:200 dilution) in the
637 solution (Jackson ImmunoResearch). Slices were mounted using Prolong Gold
638 Antifade Reagent (Invitrogen, P36930). Microscopy was carried out either with a
639 confocal microscope (Leica) or with an epifluorescence microscope (Leica), and
640 images were captured using a camera and analyzed with ImageJ.
641
642 *Statistical analysis.*
643 All statistical analyses were computed using R (The R Project, version 4.0.0). Results
644 were plotted as a mean ± s.e.m. The total number (n) of observations in each group
645 and the statistics used are indicated in figure legends. Classical comparisons between
646 means were performed using parametric tests (Student's T-test, or ANOVA for
647 comparing more than two groups when parameters followed a normal distribution
648 (Shapiro test $P > 0.05$)), and non-parametric tests (here, Mann-Whitney or Friedman)
649 when the distribution was skewed. Multiple comparisons were corrected using a
650 sequentially rejective multiple test procedure (Holm-Bonferroni correction). All
651 statistical tests were two-sided. $P > 0.05$ was considered not to be statistically
652 significant.

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688

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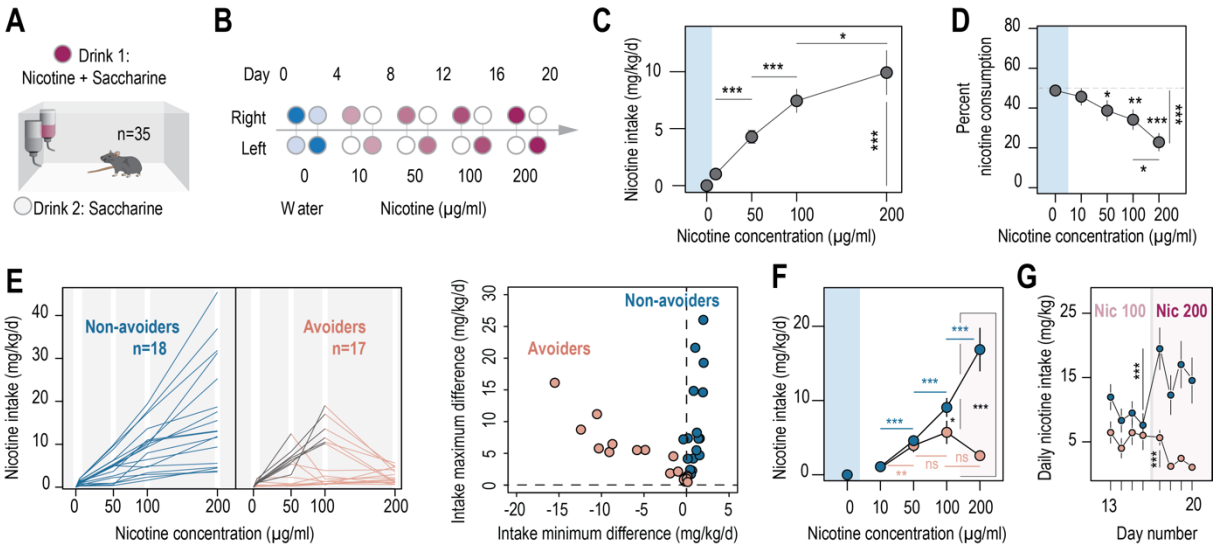
840 **Figure Legends**



841 **FIGURE 1**

842 **Figure 1: Two different profiles, avoiders and non-avoiders, emerged in WT mice**
843 **subjected to a two-bottle choice nicotine-drinking test. A.** Continuous access, two-
844 bottle choice setup. **B.** Two-bottle choice paradigm. Each dot represents a bottle and
845 is color-coded according to whether it contains water (blue or light blue), nicotine plus
846 2% saccharine (red, gradient of color intensities according to the nicotine
847 concentration), or 2% saccharine (white) solutions. The nicotine concentration in the
848 bottle increased progressively from 10 to 50, 100 and 200 µg/ml. Each condition lasted
849 four days, and the bottles were swapped every other day. **C.** Nicotine intake (mg/kg/d),
850 averaged over four days, at different nicotine concentrations (Friedman test, n = 35, df
851 = 3, $p < 0.001$ and Mann-Whitney post-hoc test with Holm-Bonferroni correction). **D.**
852 Percent nicotine consumption in WT mice for each concentration of nicotine, averaged
853 over four days (Friedman test, n = 35, df = 4, $p < 0.001$ and Mann-Whitney post-hoc
854 test with Holm-Bonferroni correction) **E.** Left, nicotine intake in individual avoiders (n =
855 17) and non-avoiders (n = 18). Right, minimum and maximum values of the difference
856 in nicotine intake between two consecutive days, for each individual. **F.** Nicotine intake

857 in avoiders and non-avoiders for each nicotine concentration, averaged over four days
858 (Mann-Whitney comparison with a Holm-Bonferroni correction). **G.** Daily nicotine
859 intake in avoiders and non-avoiders for nicotine 100 and 200 μg/ml (paired Mann-
860 Whitney). Note the drop in nicotine consumption at day 17 for avoiders. In all figure
861 panels avoiders are depicted in pinkish-orange while non-avoiders are in blue. *** $p <$
862 0.001, ** $p < 0.01$, * $p < 0.05$.

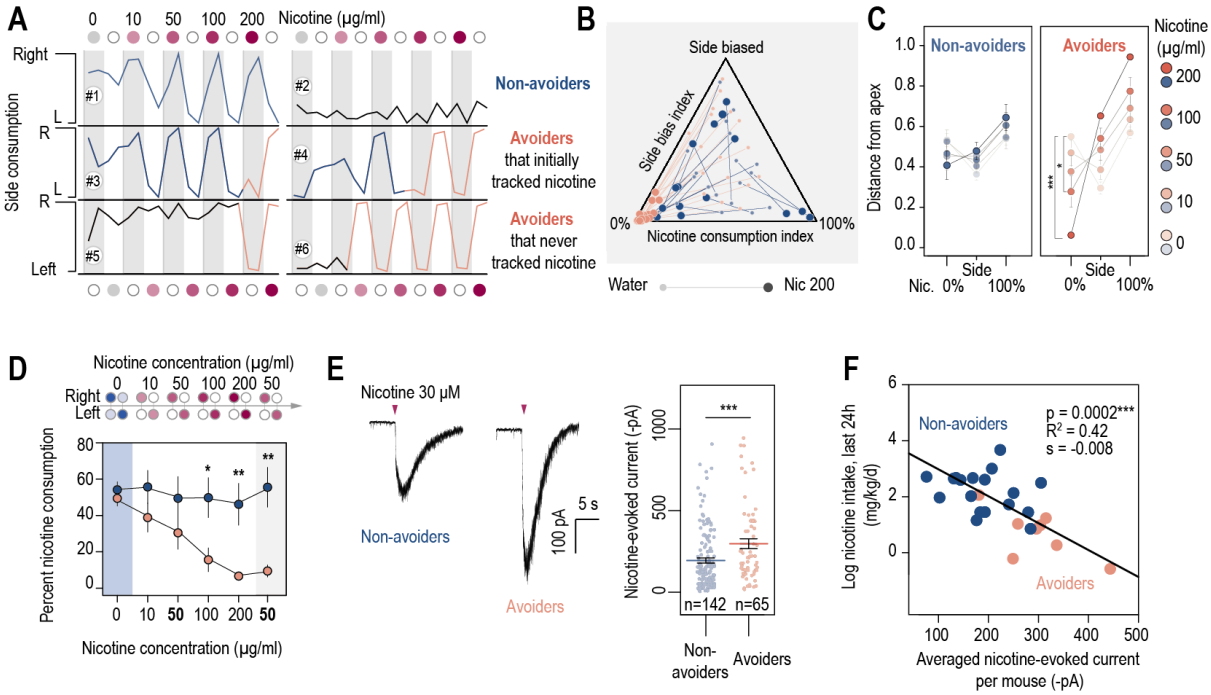


FIGURE 2

Figure 2: Nicotine intake was negatively correlated with the amplitude of the response to nicotine in IPN neurons. A. Representative examples of choice behaviors (% consumption on the right vs. left bottle) in WT mice when the right-hand side bottle contains either nicotine + saccharine (red dots, grey stripe) or saccharine only (white dots, white stripes). Mice are same as in Fig. 1. **B.** Pseudo-ternary diagram representing, for each individual (18 non-avoiders and 17 avoiders, see Fig. 1), its nicotine consumption index over its side bias index. Bottom left apex: 0% nicotine consumption (0%); Bottom right apex: 100% nicotine consumption (100%); Top apex: 100% side preference (Side biased, i.e. mice that never switch side). Small dots correspond to the habituation period (water vs. water) while bigger dots correspond to the condition with 200 µg/ml of nicotine in one bottle. Note how all avoider mice end up in the bottom left apex (0% nicotine consumption) at the end of the task. **C.** Average distance from the three apices for each condition in the task (0, 10, 50, 100 and 200 µg/ml of nicotine, color-coded from light to dark), for avoiders and non-avoiders. Only

878 avoider mice significantly changed their drinking strategy as nicotine concentration
879 increased (paired Mann-Whitney test with Holm-Bonferroni correction). **D.** Average
880 percent nicotine consumption for avoider and non-avoider mice for each concentration
881 of nicotine. The 50 µg/ml nicotine solution was presented a second time to the mice,
882 at the end of the session, for four days. (Mann-Whitney test, Holm-Bonferroni
883 correction, p(50 Nic) = 0.04, p(100 Nic) = 0.005, p(200 Nic) = 0.004). **E.** Representative
884 (left) and average (right) currents recorded in voltage-clamp mode (-60 mV) from IPN
885 neurons of non-avoiders (blue, n = 142 neurons from 18 mice, I = -194 ± 15 pA) and
886 avoiders (red, n = 65 neurons from 8 mice, I = -297 ± 30 pA) following a puff application
887 of nicotine (30 µM, 200 ms). Avoiders presented greater nicotine-evoked currents than
888 non-avoiders (Mann-Whitney test, p = 1.4e-11). **G.** Correlation between the dose
889 consumed (log scale, over the last 24 hours prior to the recording) and the averaged
890 nicotine evoked-current per mouse (-pA). In all figure panels avoiders are depicted in
891 pinkish-orange and non-avoiders in blue. *** p<0.001, ** p<0.01, * p<0.05.
892

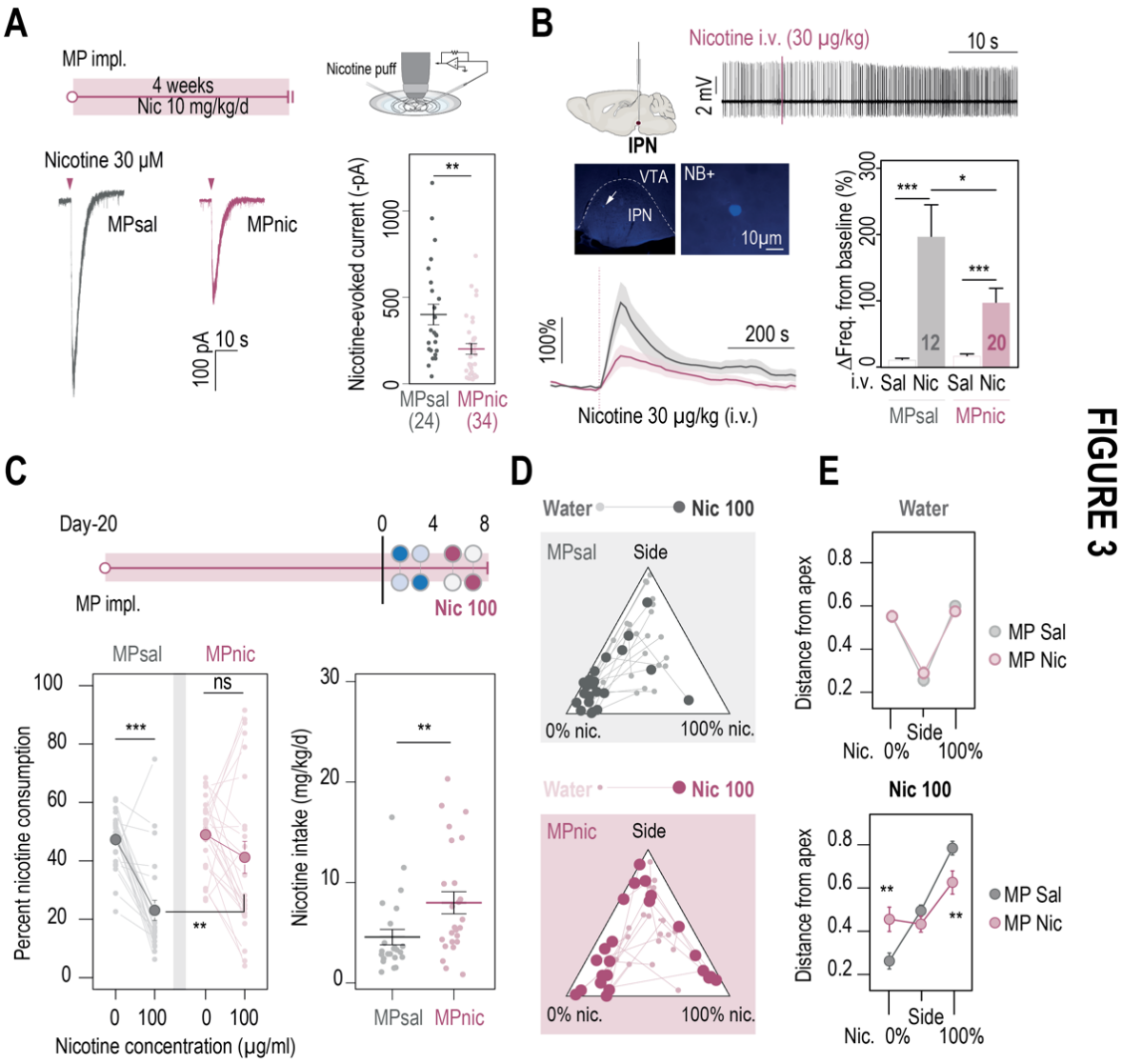


FIGURE 3

Figure 3: Chronic nicotine treatment altered both nAChR expression levels in the IPN and nicotine intake in WT mice. A. Top, passive nicotine treatment protocol. Mice were implanted subcutaneously with an osmotic minipump (MP) that continuously delivers 10 mg/kg/d of nicotine. After 4 weeks of treatment, nicotine-evoked responses in IPN neurons were recorded in whole-cell voltage-clamp mode (-60 mV) from IPN slices. Bottom, representative recordings (left) and average current amplitudes (right) following a puff application of nicotine (30 µM, 200 ms) in IPN neurons of mice treated with either saline (n = 24 neurons from 3 mice, I = -401 ± 59 pA) or nicotine (n = 34 neurons from 4 mice, I = -202 ± 37 pA). Nicotine treatment reduced the amplitude of nicotine-evoked currents in IPN neurons (Mann-Whitney test, p = 0.001). **B.** *In vivo*

904 juxtacellular recordings of nicotine-evoked responses in IPN neurons of saline- and
905 nicotine-treated animals. Top, representative electrophysiological recording of an IPN
906 neuron, during an i.v. injection of nicotine (30 µg/kg). Middle, post-recording
907 identification of neurobiotin-labeled IPN neurons by immunofluorescence. Bottom,
908 average time course and average amplitude of the change in firing frequency from
909 baseline after an i.v. injection of saline and nicotine (30 µg/kg), for IPN neurons from
910 saline- and nicotine-treated mice. Right, firing rate variation from baseline induced by
911 nicotine or saline injection in IPN neurons from saline- (n = 6) or nicotine-treated (n =
912 13) animals. Responses were decreased by chronic exposure to nicotine (p = 0.035,
913 Mann-Whitney test). All neurons were confirmed to be located within the IPN using
914 juxtacellular labeling with neurobiotin. **C.** Top, modified two-bottle choice protocol used
915 to evaluate the impact of a long-term exposure to nicotine on drug intake. Mice were
916 implanted subcutaneously with a minipump that delivered 10 mg/kg/d of nicotine
917 continuously, for 20 days before performing the modified two-bottle choice task. After
918 four days of water vs. water habituation, mice were directly exposed to a high
919 concentration of nicotine (100 µg/ml). Bottom, percent nicotine consumption and
920 nicotine intake at 0 and 100 µg/ml of nicotine, for mice under a chronic treatment of
921 nicotine or saline. The saline-treated group displayed a decrease in percent nicotine
922 consumption (n = 23, from 47.3 ± 2.0% to 23.0 ± 3.4%, p = 1.7e-05, Mann-Whitney
923 paired test), but not the the nicotine-treated group (n = 25, from 48.9 ± 2.4 to 41.2
924 ± 5.5%, p = 0.16, Mann-Whitney paired test). Overall, the saline-treated group
925 displayed a lower percent nicotine consumption (p = 0.003, Mann Whitney) and lower
926 nicotine intake than the nicotine-treated group (p = 0.004, Mann-Whitney). **D.** Pseudo-
927 ternary diagrams representing each saline- and nicotine-treated mouse for its nicotine
928 consumption index over its side bias index. Small dots correspond to the habituation

929 period (water vs. water) and bigger dots to the condition with 100 µg/ml of nicotine in
930 one bottle. **E.** Average distance from each apex in the water vs. water (top) and water
931 vs. nicotine 100 µg/ml conditions (bottom). Saline-treated, but not nicotine-treated mice
932 developed a strategy to avoid nicotine ($p_{Sacc} = 0.013$, $p_{Side} = 0.27$ $p_{Nic} = 0.013$, Mann-
933 Whitney test with Holm-Bonferroni correction). In all figure panels nicotine-treated
934 animals are displayed in red and saline-treated (control) animals in grey.
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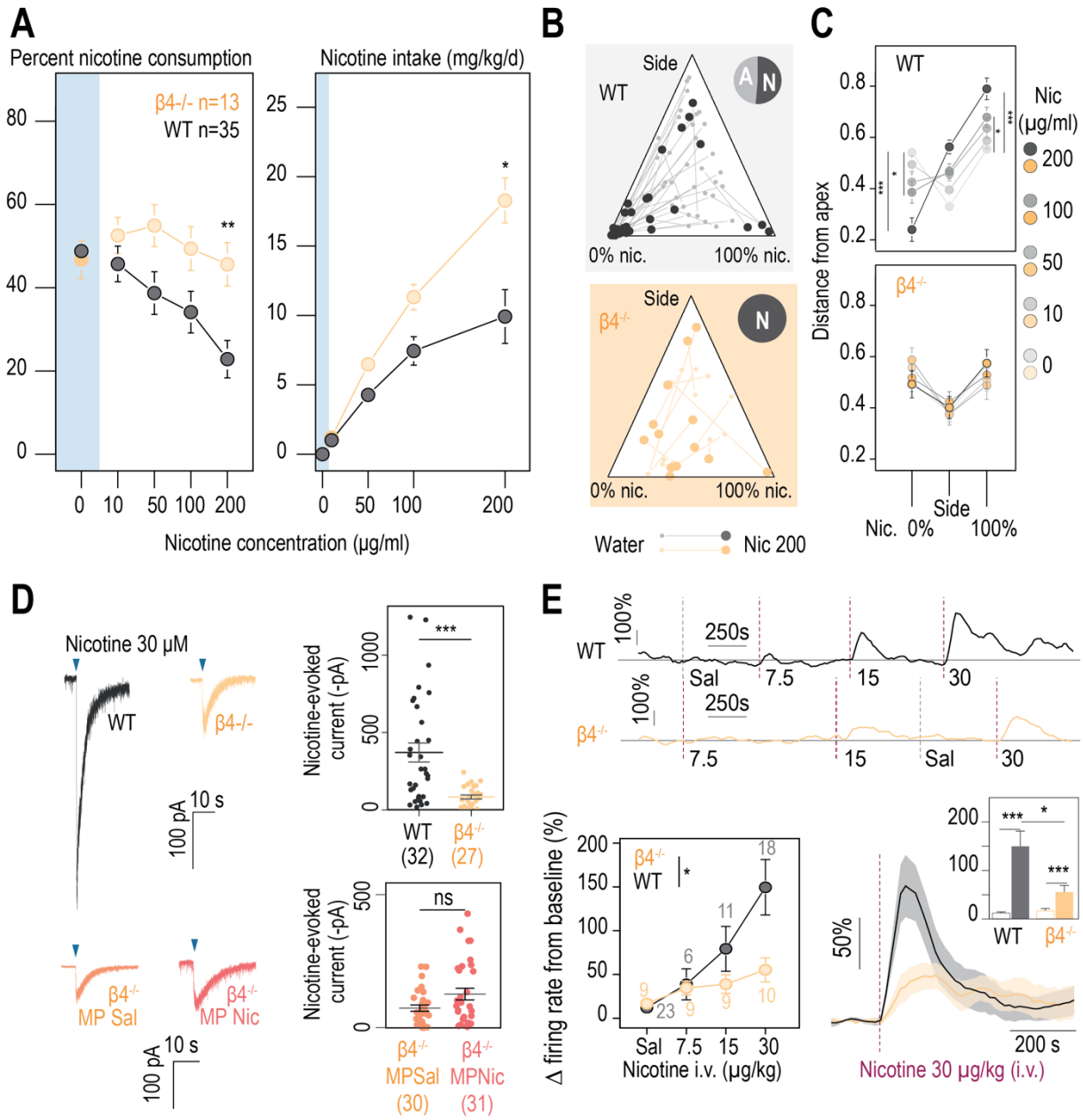

936 **FIGURE 4**

937 **Figure 4: β4-containing nicotinic receptors are essential for triggering nicotine**
938 **aversion in mice. A.** Left, average percent nicotine consumption in WT and β4$^{-/-}$ mice
939 for each concentration of nicotine in the two-bottle choice task. WT mice had lower
940 percent nicotine consumption than β4$^{-/-}$ mice (Mann-Whitney test with Holm-Bonferroni
941 correction). WT mice decreased their percent nicotine consumption throughout the
942 task (Friedman test, n = 35, df = 4, $p < 0.001$ and Mann-Whitney post-hoc test with
943 Holm-Bonferroni correction) while β4$^{-/-}$ mice displayed a stable percent nicotine

944 consumption (Friedman test, n = 13, df = 4, p = 0.11). Right, average nicotine intake
945 (mg/kg/d) in $\beta4^{-/-}$ and WT mice for the different concentrations of nicotine (Friedman
946 test, n = 35, df = 3, p < 0.001 and Mann-Whitney post-hoc test with Holm-Bonferroni
947 correction). $\beta4^{-/-}$ mice consumed more nicotine than WT mice (Mann-Whitney test). **B.**
948 Ternary diagram representing each WT and $\beta4^{-/-}$ individual for its nicotine consumption
949 index over its side bias index. Small dots correspond to the habituation period (water
950 vs. water) and bigger dots to the condition with 200 µg/ml of nicotine in one bottle.
951 Inserts: pie charts illustrating the proportion of avoiders (A, light grey) and non-avoiders
952 (N, dark grey) for each genotype at the end of the task. Note the absence of avoiders
953 in $\beta4^{-/-}$ mice. **C.** Average distance from each apex at 0, 10, 50, 100 and 200 µg/ml of
954 nicotine (paired Mann-Whitney test with Holm-Bonferroni correction, p(Sacc 0-200) =
955 0.0002, p(Sacc 0-100) = 0.03; p(Nic 0-200) = 0.0002, p(Sacc 0-100) = 0.03). **D.** Left,
956 representative currents following a puff application of nicotine (30 µM, 200 ms) in IPN
957 neurons from naive WT and $\beta4^{-/-}$ mice, or from saline-treated (orange) and nicotine-
958 treated (dark orange) $\beta4^{-/-}$ mice. Right, average nicotine-evoked currents recorded in
959 IPN neurons from naïve WT (n = 32 neurons from 5 mice, I = -370 $\pm$ 61 pA) and $\beta4^{-/-}$
960 (n = 27 neurons from 4 mice, I = -83 $\pm$ 13 pA) mice, and from $\beta4^{-/-}$ chronically treated
961 with either saline (Sal, n = 30 neurons from 6 mice, I = -72 $\pm$ 11 pA) or nicotine (Nic, n
962 = 31 neurons from 5 mice, I = -123 $\pm$ 21 pA). $\beta4^{-/-}$ mice presented a large decrease in
963 nicotine-evoked currents (Mann-Whitney test, p = 4e-05). Nicotine treatment did not
964 alter nicotine-evoked currents in IPN neurons of $\beta4^{-/-}$ mice (Mann-Whitney test, p =
965 0.15). **E.** Juxtacellular recordings of nicotine-evoked responses in IPN neurons in naive
966 WT and $\beta4^{-/-}$ mice. Top, representative example of the variation in firing frequency of
967 an IPN neuron, following repeated i.v. injections of nicotine at 7.5, 15 and 30 µg/kg, in
968 WT and $\beta4^{-/-}$ mice. Bottom left, dose-dependent change in firing rate from baseline

969 following i.v. injections of nicotine ($p < 0.05$). All recorded neurons were neurobiotin-
970 labelled to confirm their location within the IPN. Bottom right, average nicotine-evoked
971 responses at 30 μg/kg of nicotine in IPN neurons from WT and $\beta4^{-/-}$ mice. Insert,
972 average amplitude of the change in firing frequency from baseline after an i.v. injection
973 of saline and nicotine (30μg/kg), for IPN neurons from WT ($n = 18$) and $\beta4^{-/-}$ ($n = 7$)
974 mice. Nicotine-induced responses were smaller in $\beta4^{-/-}$ than in WT mice ($p < 0.001$,
975 Mann Whitney test). In all figure panels WT animals are depicted in grey and $\beta4^{-/-}$ mice
976 in yellow. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.
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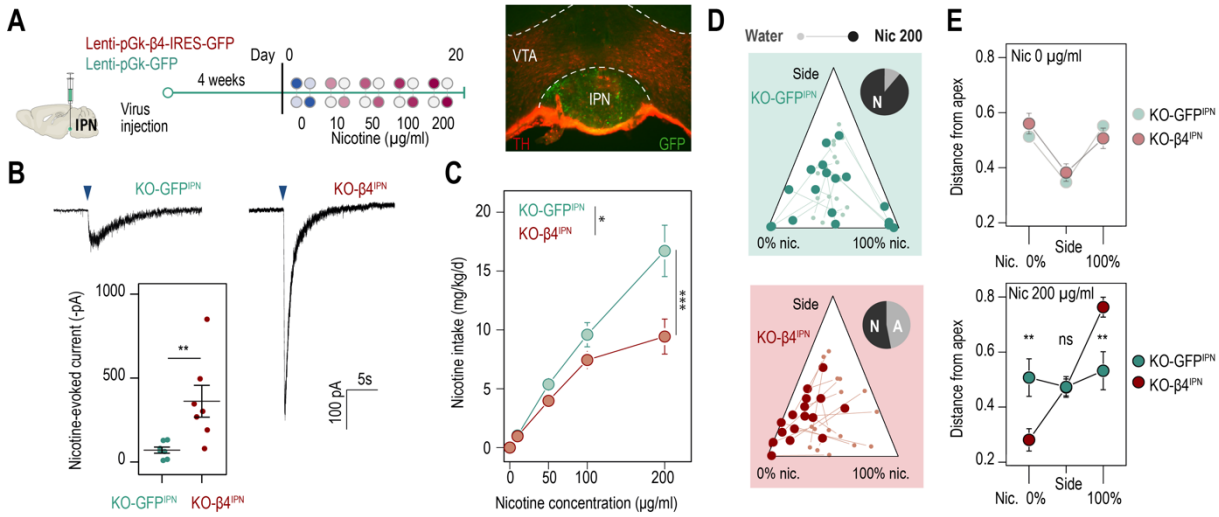

978 **FIGURE 5**

979 **Figure 5: β4-containing nAChRs of the IPN are involved in the control of nicotine**
980 **consumption and in aversion to nicotine in mice. A.** Protocol: stereotaxic
981 transduction of the β4 subunit together with GFP (or GFP alone in control mice) in the
982 IPN of $\beta4^{-/-}$ mice, and subsequent two-bottle choice task. Right: coronal section
983 highlighting proper viral transduction of lenti-pGK-β4-IRES-GFP in the IPN. **B.**
984 Validation of the re-expression using whole-cell patch-clamp recordings.
985 Representative currents and average responses following a puff application of nicotine
986 (30 µM, 200 ms) on IPN neurons from $\beta4^{-/-}$ mice transduced in the IPN with either lenti-
987 pGK-β4-IRES-GFP (KO-$\beta4^{IPN}$, n = 7 neurons from 2 mice, I = -362 ± 95 pA) or lenti-
988 pGK-GFP (KO-GFP^{IPN}, n = 7 neurons from 1 mouse, I = -71 ± 18 pA; Mann-Whitney
989 test, p = 0.004). **C.** Average nicotine intake was lower in KO-$\beta4^{IPN}$ than in KO-GFP^{IPN}
990 (two-way repeated measure; ANOVA: genotype x dose interaction, F[3, 99] = 6.3, ***p
991 < 0.001; main effect of dose, F[3, 99] = 69.1 ***p < 0.001, effect of genotype, F[1, 33]
992 = 6.637, *p = 0.015). **D.** Ternary diagram representing each $\beta4^{-/-}$ mouse, transduced
993 with either β4 or GFP, and illustrating its nicotine consumption index over its side bias
994 index. Small dots correspond to the habituation period (water vs. water) and bigger
995 dots to the condition with 200 µg/ml of nicotine in one bottle. Inserts: pie charts

illustrating the proportion of avoiders (A, light grey) and non-avoiders (N, dark grey) for each condition at the end of the task. **E.** Average distance from each apex during the two-bottle choice task at 0 and 200 µg/ml of nicotine, for KO-β4^{IPN} and KO-GFP^{IPN} mice ($p_{Sacc} = 0.007$, $p_{Nic} = 0.008$, Mann-Whitney test with Holm-Bonferroni correction). In all figure panels KO-β4^{IPN} mice are depicted in red and KO-GFP^{IPN} mice (controls) in green. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.