



Article

Lateral Habenula 5-HT_{2C} Receptor Function Is Altered by Acute and Chronic Nicotine Exposures

Cristiano Bombardi ¹, Francis Delicata ², Claudio Tagliavia ¹, Annamaria Grandis ¹, Massimo Pierucci ², Antonella Marino Gammazza ³, Maurizio Casarrubea ⁴, Philippe De Deurwaerdère ⁵ and Giuseppe Di Giovanni ^{2,6,*}

- ¹ Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50, 40064 Ozzano dell'Emilia, Italy; cristiano.bombardi@unibo.it (C.B.); claudio.tagliavia2@unibo.it (C.T.); annamaria.grandis@unibo.it (A.G.)
- ² Laboratory of Neurophysiology, Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, MSD2080 Msida, Malta; francis.delicata.06@um.edu.mt (F.D.); Massimo.pierucci@um.edu.mt (M.P.)
- ³ Section of Human Anatomy, Department of Biomedicine, Neuroscience and Advanced Diagnostics (BiND), University of Palermo, 90127 Palermo, Italy; antonella.marinogammazza@unipa.it
- ⁴ Laboratory of Behavioral Physiology, Human Physiology Section "Giuseppe Pagano", Department of Biomedicine, Neuroscience and Advanced Diagnostics (BiND), University of Palermo, 90127 Palermo, Italy; maurizio.casarrubea@unipa.it
- ⁵ Unité Mixte de Recherche 5287, Centre National de la Recherche Scientifique, 146 rue Léo Saignat, B.P.281, CEDEX, F-33000 Bordeaux, France; philippe.de-deurwaerdere@u-bordeaux.fr
- ⁶ School of Biosciences, Cardiff University, Cardiff CF10 3AX, UK
- * Correspondence: giuseppe.digiovanni@um.edu.mt or digiovannig@cardiff.ac.uk; Tel.: +356-23402776



Citation: Bombardi, C.; Delicata, F.; Tagliavia, C.; Grandis, A.; Pierucci, M.; Marino Gammazza, A.; Casarrubea, M.; De Deurwaerdère, P.; Di Giovanni, G. Lateral Habenula 5-HT_{2C} Receptor Function Is Altered by Acute and Chronic Nicotine Exposures. *Int. J. Mol. Sci.* **2021**, *22*, 4775.
<https://doi.org/10.3390/ijms22094775>

Academic Editor: Eiko Nakamaru-Ogiso

Received: 1 April 2021
Accepted: 27 April 2021
Published: 30 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Serotonin (5-HT) is important in some nicotine actions in the CNS. Among all the 5-HT receptors (5-HTRs), the 5-HT_{2C}R has emerged as a promising drug target for smoking cessation. The 5-HT_{2C}Rs within the lateral habenula (LHb) may be crucial for nicotine addiction. Here we showed that after acute nicotine tartrate (2 mg/kg, i.p.) exposure, the 5-HT_{2C}R agonist Ro 60-0175 (5–640 µg/kg, i.v.) increased the electrical activity of 42% of the LHb recorded neurons in vivo in rats. Conversely, after chronic nicotine treatment (6 mg/kg/day, i.p., for 14 days), Ro 60-0175 was incapable of affecting the LHb neuronal discharge. Moreover, acute nicotine exposure increased the 5-HT_{2C}R-immunoreactive (IR) area while decreasing the number of 5-HT_{2C}R-IR neurons in the LHb. On the other hand, chronic nicotine increased both the 5-HT_{2C}R-IR area and 5-HT_{2C}R-IR LHb neurons in the LHb. Western blot analysis confirmed these findings and further revealed an increase of 5-HT_{2C}R expression in the medial prefrontal cortex after chronic nicotine exposure not detected by the immunohistochemistry. Altogether, these data show that acute and chronic nicotine exposure differentially affect the central 5-HT_{2C}R function mainly in the LHb and this may be relevant in nicotine addiction and its treatment.

Keywords: Ro 60-0175; nucleus accumbens; dorsal raphe nucleus; addiction; depression; medial prefrontal cortex; dentate gyrus; striatum; ventral tegmental area; substantia nigra pars compacta; single cell-extracellular recording

1. Introduction

Serotonin 2C receptors (5-HT_{2C}Rs) are widely distributed in the mammalian brain [1], with the highest concentration in the choroid plexus followed by the cortex, the substantia nigra, the globus pallidus, the olfactory system, the hippocampus and hypothalamus, the amygdala, and some thalamic nuclei [2]. 5-HT_{2C}Rs are preferentially expressed at the postsynaptic level, although they are also present presynaptically on neuron terminals other than 5-HT neurons [1,2].

A large body of evidence shows that the 5-HT_{2C}R is central in drug addiction, including nicotine dependence and relapse (see [2–4]). Activation of 5-HT_{2C}Rs decreased nicotine-induced locomotion, nicotine self-administration and reinstatement of nicotine-seeking, and the discriminative stimulus properties of nicotine [5–11]. Nevertheless, enhancing 5-HT_{2C}R activation has a large inhibitory influence on different appetitive and consummatory behaviors dampening for example food and cocaine reinforcing effects [12,13]. Promising preclinical and clinical trial results on 5-HT_{2C}R agonism as a new smoking cessation treatment, have arisen from lorcaserin [14,15], approved first by the US Food and Drug Administration (FDA) as an anti-obesity drug that unfortunately has been recently withdrawn over cancer concerns [16]. Nevertheless, the 5-HT_{2C}R still represents an important drug target for the treatment of nicotine addiction and a better understanding of the mechanisms linking nicotine and 5-HT_{2C}R agonist efficacy is required.

Nicotine addiction involves functional changes in several neurochemical systems [17,18] and neurobiological networks [19] that are critically regulated by the ventral tegmental area (VTA) dopaminergic neurons, similar to numerous drugs of abuse [20]. Serotonin is known to modulate dopaminergic function [21,22]. Compelling evidence shows that 5-HT_{2C}R activation decreases the activity of the VTA dopaminergic neurons [23–25] presumably by activating the GABAergic VTA interneurons [26], thereby leading to a decrease of accumbal dopamine (DA) release under basal [27] and stimulated [28,29] conditions. The activation of the 5-HT_{2C}Rs might reduce rewarding nicotine effects by halting the elevation of accumbal DA release and VTA cell firing elicited by chronic treatment with nicotine [17,30,31]. On the other hand, several brain regions harbor 5-HT_{2C}R that may act on the activity of the dopaminergic neurons of the VTA, or even act independently of net changes of dopaminergic function as in the case of cocaine, but alter the behavioral outputs [22]. A broader conception of the link between nicotine and 5-HT_{2C}R is supported by data reporting that chronic nicotine treatment (0.4 mg/kg for 5 days in mice) reduced [³H]mesulergine labeling to 5-HT_{2C}Rs in subregions of the medial prefrontal cortex (mPFC), and nicotine paired with environmental context produced more robust changes in the prelimbic cortex [8]. The same authors showed that 3-day nicotine withdrawal from a similar drug treatment induced a reduction in [³H]mesulergine binding to 5-HT_{2C}Rs in the ventral dentate gyrus (DG), with no change in 5-HT_{2C}R mRNA in the hippocampus (HP) but decreased 5-HT_{2C}R mRNA editing in mice [32]. Although parcellar, these data point out possible changes of the reactivity of 5-HT_{2C}Rs in some brain regions associated with modification of their pattern of expression in nicotine addiction.

The lateral habenula (LHb), a small epithalamic structure has recently attracted attention as a possible area involved in the 5-HT_{2C}R effects in the CNS [2,33]. LHb conveys negative motivational signals that could be dependent on its inhibitory control over dopaminergic brain areas [34]. The LHb has also been indicated as a hub for nicotine effects, i.e., nicotine excites LHb neurons *in vivo* [35] and *in vitro* [36] and acute and chronic nicotine-induced anxiety is counteracted by LHb lesion [37,38]. A role of 5-HT_{2C}Rs in the modulation of LHb neuronal activity has been previously shown, reporting upon their activation excitatory responses *in vitro* [39] and both excitatory [33,40] and inhibitory responses *in vivo* [33]. The mixed responses are likely due to the 5-HT_{2C}Rs expressed within the LHb and other brain areas [33]. We hypothesized that acute and chronic nicotine exposure alters the reactivity of LHb neurons to the stimulation of 5-HT_{2C}R associated with a reorganization of 5-HT_{2C}R expression.

In the present study, the electrophysiological effect of intravenous (*i.v.*) administration of the 5-HT_{2C}R agonist Ro 60-0175 [41] was studied in rats using extracellular single-cell recordings *in vivo* to probe the functional responsiveness of LHb neurons in acute or chronic nicotine treated rats. The electrophysiological study was furthered by a detailed immunohistochemical analysis of 5-HT_{2C}R expression in different brain regions possibly involved in nicotine addiction including the LHb. This study was extended by a more quantitative analysis using Western blotting (WB) of 5-HT_{2C}R expression in tissue from the

LHb, the mPFC, hippocampal DG, the nucleus accumbens (NAc), the striatum (ST), the VTA, the substantia nigra pars compacta (SNc), and the dorsal raphe nucleus (DRN).

2. Results

2.1. Effect of Systemic Administration of 5-HT_{2C}R Agonist Ro 60-0175 on the Firing Rate of LHb Neurons of Acute and Chronic-Treated Rats In Vivo

2.1.1. Firing Characteristics of Spontaneously Active LHb Neurons In Vivo

Forty-nine spontaneously active neurons were recorded extracellularly from the LHb anesthetized adult male Sprague Dawley rats acutely or chronically exposed to nicotine. Their electrophysiological characteristics were similar to that which we previously reported [33,42], with an average firing rate of 11.4 ± 1.06 Hz and a waveform duration of 1.1 ± 0.02 ms. The majority of neurons had a biphasic waveform (95.9%) and an irregular firing pattern (81.6%). The overall average coefficient of variation (CV) was 0.77 ± 0.04 .

2.1.2. Systemic Administration of Ro 60-0175 on the Firing Rate of LHb Neurons In Vivo in Acute and Chronic-Treated Rats

Intravenous administration of Ro 60-0175 (5–640 μ g/kg, iv), a selective 5-HT_{2C}R agonist, one hour after acute nicotine hydrogen tartrate salt treatment (2 mg/kg; i.p. equivalent to 0.7 mg/kg nicotine free base) induced a significant increase of LHb neuronal firing rate in 42% of recorded neurons and reached peak effect at 320 μ g/kg ($44.8 \pm 13.7\%$, one-way ANOVA with repeated measures, followed by Tukey's post hoc test, $F(2,24) = 10.407$, $p = 0.01$, Figure 1A,B). The remaining cells did not alter their firing rate at any point during the dose-response treatment. The increase in firing rate observed in the acute nicotine-treated group was not different from that recorded in naïve nicotine [34] at any of the doses tested (two-way ANOVA followed by Tukey's test, $p = 0.9$) (not shown). Ro 60-0175 (5–640 μ g/kg, iv) administration one hour after nicotine-treatment (2 mg/kg; i.p.) was not capable of altering the firing rate of the LHb neurons in chronic nicotine-treated rats (14 days, at 6 mg/kg/day i.p.; equivalent to 2.1 mg/kg nicotine free base) (Figure 1C,D).

2.2. Effect of Systemic Administration of Acute and Chronic Nicotine on 5-HT_{2C}R Immunohistochemical Expression in the LHb in Rats

To evaluate the effect of systemic administration of acute and chronic nicotine on 5-HT_{2C}R immunohistochemical expression in the LHb we used naïve nicotine (NN, $n = 8$), acute nicotine (AN, $n = 8$), and chronic nicotine (CN, $n = 8$) treated with the same protocol of the electrophysiological experiments. Specifically, the rats received 1 h before the sacrifice, nicotine vehicle or nicotine (2 mg/kg; i.p.). CN rats received for 14 days nicotine 6 mg/kg/day i.p.

2.2.1. Immunoperoxidase Experiments

5-HT_{2C}R immunolabeling was especially found in cell bodies and neuropil. Dendritic immunoreactive profiles could be observed in the LHb. The neuropil immunoreactivity, consisting only of diffuse staining, was not associated with any identifiable neuronal structure.

The LHb of nicotine naïve rats demonstrated a low level of immunostaining for the 5-HT_{2C}R (Figure 2A,B; Table 1). This area had a medium density of 5-HT_{2C}R-immunopositive neurons whereas the neuropil labeling was low (Figure 2B; Table 1). The LHb of acute nicotine rats exhibited strong immunostaining for the 5-HT_{2C}R (Figure 2C,D; Table 1). Interestingly, the neuropil was primarily immunostained whereas the density of immunostained cells was low (Figure 2D; Table 2). The LHb of chronic nicotine rats demonstrated prominent immunostaining for the 5-HT_{2C}R (Figure 2E,F; Table 1). This area not only contained a high density of 5-HT_{2C}R-IR neurons but also exhibited strong neuropilar immunostaining (Figure 2F; Table 1).

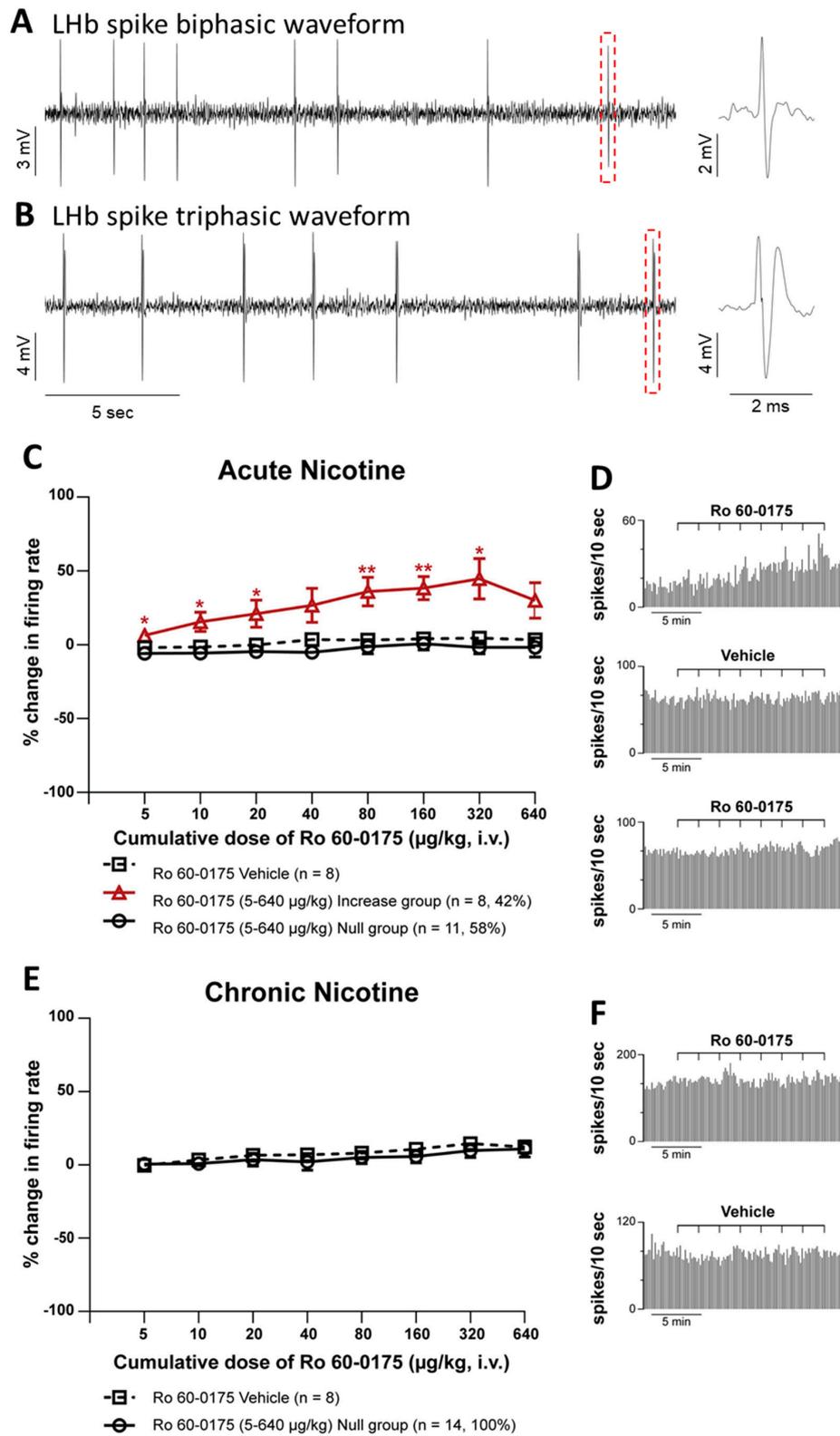


Figure 1. Effect of systemic administration of Ro 60-0175, a selective 5-HT_{2C}R agonist, on LHB neuronal firing, after the systemic administration of nicotine, in vivo in anesthetized rats. (A,B) Raw traces of two typical LHB neurons. Enlargements show a single detected spike with a biphasic waveform (top trace, A) and triphasic waveform (bottom trace, B). The majority of neurons recorded had a biphasic waveform (95.9%). (C) A dose-response curve of Ro 60-0175 (5–640 µg/kg, iv) after a

single acute treatment of nicotine. Data are shown as the mean % change in firing rate \pm SEM. The majority of neurons ($n = 11$, 58%) did not respond to agonist administration, at any dose (vehicle $n = 8$). The remaining neurons ($n = 8$; shown 42%) responded with an increase in firing rate, with a peak effect at 320 $\mu\text{g}/\text{kg}$ (44.8 ± 13.7). (D) Typical rate histograms of single neuronal recordings that showed an increase in firing (top) and no change in firing (bottom) after Ro 60-0175 and a control neuron (middle) after Ro 60-0175 vehicle administration in acute nicotine-treated rats. (E) A dose-response curve of Ro 60-0175 (5–640 $\mu\text{g}/\text{kg}$, iv; $n = 14$) after chronic treatment of nicotine compared to the effect of vehicle ($n = 8$). (F) Typical rate histograms of single neuronal recordings showed no change in firing after Ro 60-0175 (top) and a control neuron (bottom) after Ro 60-0175 vehicle administration. Data are shown as the mean % change in firing rate \pm SEM. All neurons failed to respond to agonist administration. Independent sample *t*-test, $p > 0.05$ vs Vehicle at every dose. (D) Typical rate histograms of single neuronal recordings showed no change in firing (top) after Ro 60-0175 and a control neuron (bottom) after Ro 60-0175 vehicle administration. One-way ANOVA with repeated measures, followed by Tukey's post hoc test, * $p < 0.05$ vs. Vehicle, $p^{**} < 0.005$ vs. Vehicle.

LATERAL HABENULA

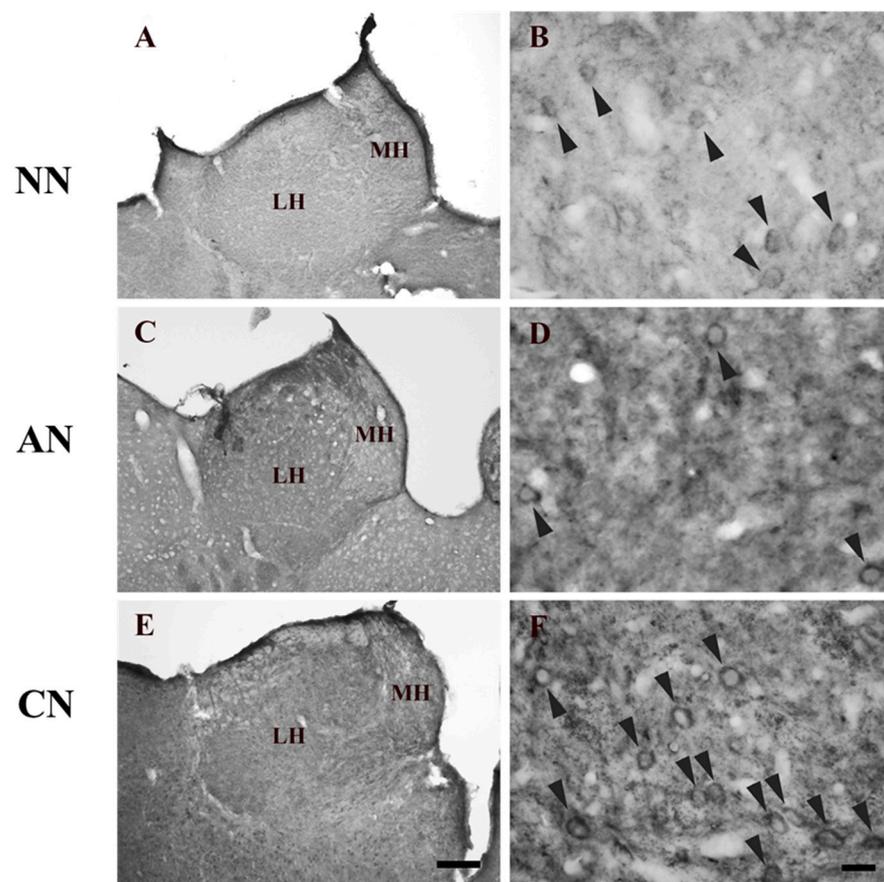


Figure 2. Brightfield photomicrographs of coronal sections showing the distribution of 5-HT_{2C} receptors (5-HT_{2C}R) immunoreactivity in the lateral habenula (LHb) of nicotine-naïve (NN; (A,B)), acute nicotine (AN; (C,D)), and chronic nicotine (CN; (E,F)). Note the high 5-HT_{2C}R immunoreactivity in the LHb of acute and chronic nicotine rats (C,E). However, acute nicotine rats show the lowest density of immunoreactive somata. See text and Tables 1 and 2 for explanations. Abbreviations: LHb, lateral habenula; MH, medial habenula. Scale bar = 200 μm in (E) (applies to (A,C,E)) and 15 μm in (F) (applies to (B,D,F)). Naïve nicotine (NN, $n = 8$), acute nicotine (AN, $n = 8$) and chronic (CN, $n = 8$). Arrowheads indicate immunoreactive somata.

Table 1. 5-HT_{2C}R-IR somata density and colocalization of HuC/D with 5-HT_{2C}R in the rat lateral habenula.

Lateral Habenula (LHb)	Naïve Nicotine (NN)	Acute Nicotine (AN)	Chronic Nicotine (CN)
Density 5-HT _{2C} R-IR neurons	49.2 ± 11.4	18.1 ± 5.1 ^{*,#}	95.2 ± 21.1 ^{*,§}
% area covered by 5-HT _{2C} R-IR PGP 9.5-IR neurons	40.3 ± 12.4	68.2 ± 6.1 [*]	67.1 ± 24.1 [*]
PGP 9.5-IR neurons	624	655	638
PGP 9.5-IR/5-HT _{2C} R-IR	64	35	154
% of 5-HT _{2C} R-IR	9.3% (64/688)	5.1% (35/690) ^{*,#}	19.4% (154/792) ^{*,§}

The density of 5-HT_{2C}R-immunoreactive somata is expressed as the mean/mm² ± standard deviation. ^{*} *p* < 0.05, versus naïve nicotine (NN) and [§] *p* < 0.05 versus acute nicotine (AN) [#] *p* < 0.05 versus chronic nicotine (CN). NN data are from our recent study [33].

Table 2. 5-HT_{2C}R-IR somata density in mPFC, hippocampal DG, the nucleus accumbens (NAc), the striatum (ST), the VTA, the substantia nigra pars compacta (SNc), and the dorsal raphe nucleus (DRN).

Medial Prefrontal Cortex (mPFC) (Layers V and VI)	Naïve Nicotine (NN)	Acute Nicotine (AN)	Chronic Nicotine (CN)
5-HT _{2C} R-IR neurons	1273.8 ± 239.3	1324.1 ± 219.4	1371.2 ± 285.5
% area covered by 5-HT _{2C} R-IR	52.7 ± 10.4	52.3 ± 12.2	50.1 ± 13.8
Dentate Gyrus (DG) (polymorphic layer)	NN	AN	CN
5-HT _{2C} R-IR neurons	30.1 ± 8.6	33.6 ± 9.9	34.1 ± 8.7
% area covered by 5-HT _{2C} R-IR	33.4 ± 10.9	32.9 ± 9.6	34.8 ± 8.4
Nucleus Accumbens (NAc)	NN	AN	CN
5-HT _{2C} R-IR neurons	1123.8 ± 123.8	1128.9 ± 129.5	1134.6 ± 120.7
% area covered by 5-HT _{2C} R-IR	43.5 ± 10.6	42.1 ± 10.9	41.8 ± 11.5
Striatum (ST)	NN	AN	CN
5-HT _{2C} R-IR neurons	1121.5 ± 225.9	1129 ± 227.4	1126.3 ± 220.9
% area covered by 5-HT _{2C} R-IR	52.5 ± 14.5	53.3 ± 13.9	51.9 ± 13.6
Ventral Tegmental Area (VTA)	NN	AN	CN
5-HT _{2C} R-IR neurons	1135.9 ± 224.6	1138.1 ± 229.4	1139.9 ± 225.6
% area covered by 5-HT _{2C} R-IR	67.8 ± 15.9	65.1 ± 12.8	64.8 ± 11.4
Substantia Nigra Pars Compacta (SNc)	NN	AN	CN
5-HT _{2C} R-IR neurons	160.3 ± 20.1	162.5 ± 21.8	169 ± 22.4
% area covered by 5-HT _{2C} R-IR	40.8 ± 11.7	40.9 ± 12.4	41.3 ± 11.1
Dorsal raphe Nucleus (DRN)	NN	AN	CN
5-HT _{2C} R-IR neurons	292.8 ± 40.1	294.1 ± 38.6	300.3 ± 39.2
% area covered by 5-HT _{2C} R-IR	31.2 ± 10.8	32.3 ± 11.4	32.9 ± 11.8

The density of 5-HT_{2C} receptors-immunoreactive (5-HT_{2C}R-IR) somata is expressed as the mean/mm² ± standard deviation.

2.2.2. Double Immunofluorescence Studies

The double immunofluorescence analysis consisted of the colocalization of the 5-HT_{2C}R with PGP 9.5 (pan-neuronal marker) in the LHb (Figure 3). Since pan-neuronal markers stained every neuron, we could estimate the percentage of 5-HT_{2C}R-immunoreactive somata distributed in the LHb. As reported in Table 1, the proportion of 5-HT_{2C}R-IR neurons to the total neurons was significantly higher in chronic nicotine than in drug naïve and acute nicotine rats. In addition, the proportion of 5-HT_{2C}R-IR neurons was significantly higher in drug naïve than in acute nicotine rats. These data are in agreement with those obtained using immunoperoxidase experiments (Figure 2).

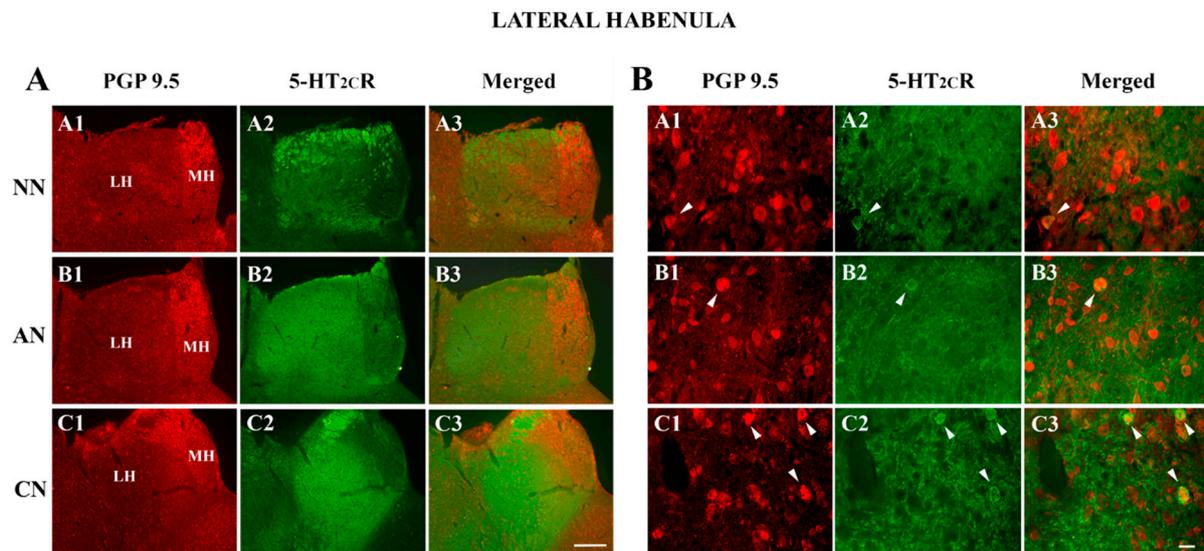


Figure 3. Colocalization of PGP 9.5 (a neuron-specific protein) with 5-HT_{2c}R receptors (5-HT_{2c}Rs) in the lateral habenula (LHb) of naïve nicotine (A1–A3), acute nicotine (B1–B3), and chronic nicotine (C1–C3) rats. **(A,B)** Double immunofluorescence images showing HuC/D in green (left column pictures; A1, B1, C1), 5-HT_{2c}R in red (middle column pictures; A2, B2, C2), and colocalization of HuC/D with 5-HT_{2c}R in yellow (right column merging pictures; A3, B3, C3). **(A)**. Note the high 5-HT_{2c}R immunoreactivity in the LHb of acute nicotine rats. **(B)**. Note that chronic nicotine rats contain the highest number of double-labeled cells (arrowheads). See text and Table 1 for explanations. Abbreviations: NN, naïve nicotine; AN, acute nicotine; CN, chronic nicotine; LHb, lateral habenula; MH, medial habenula. Scale bar = 200 μ m in **(A)** C3 (applies to **(A)** A1–C3) and scale bar = 20 μ m in **(B)** D3 (applies to **(B)** A1–C3). Naïve nicotine (NN, n = 8), acute nicotine (AN, n = 8) and chronic (CN, n = 8). Arrowheads indicate immunoreactive somata.

2.3. Effect of Systemic Administration of Acute and Chronic Nicotine on 5-HT_{2c}R Levels in the mPFC, DG, NAc, ST, VTA, SNc, and DRN Measured by Western Blotting

5-HT_{2c}R levels were quantified in lysates of the LHb, mPFC, HP, midbrain (MB), striatum (ST), and cerebellum (CR) of naïve nicotine rats (control group, NN, n = 5), acute nicotine (AN, n = 5), and chronic nicotine-treated rats (CN, n = 5) treated with the same protocol of the electrophysiological and immunohistochemistry experiments. As showed in Figure 4, 5-HT_{2c}R levels were significantly increased in LHb, PFC, and HP from chronically treated rats compared to all the other groups ($p < 0.05$). Instead, the receptor levels were significantly increased in the MB of the AN group compared to the CN group and the control group (Figure 4; $p < 0.05$). No significant change for 5-HT_{2c}R levels was found in the ST and CR.

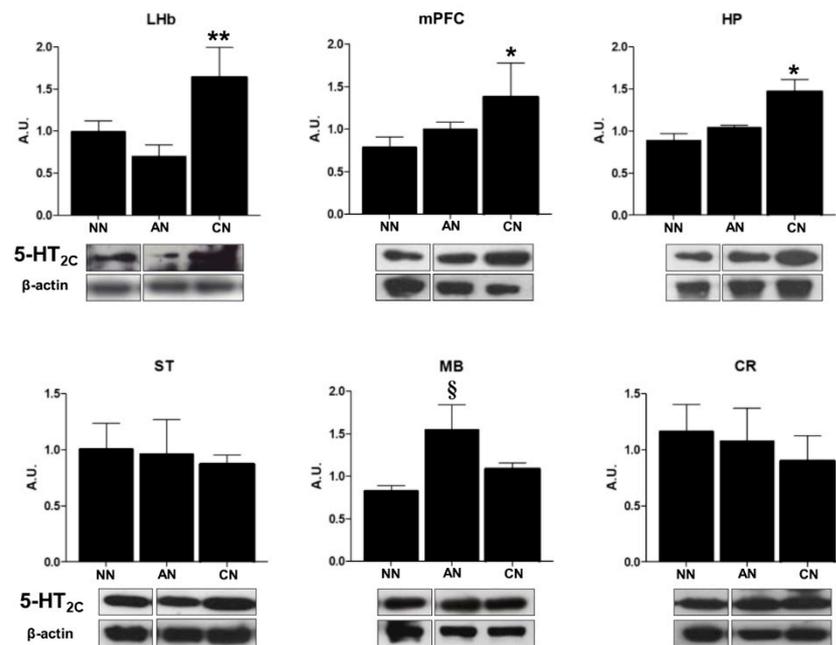


Figure 4. Representative histograms and cropped blots of 5-HT_{2C} (5-HT_{2C}) receptors levels in the lateral habenula (LHb), medial prefrontal cortex (mPFC), hippocampus (HP), striatum (ST), midbrain (MB), and cerebellum (CR) of naïve nicotine (NN), acute nicotine (AN), and chronic nicotine (CN) rats. The gels were run under the same experimental conditions and β -actin was used as an internal control. One-way ANOVA with repeated measures, followed by Tukey's post hoc test, * $p < 0.05$ vs. NN; $p < 0.001$ vs. D. ** $p < 0.05$ vs. D; $p < 0.001$ vs. A. § $p < 0.05$ vs. D and C. A.U.: arbitrary unit. Naïve nicotine (NN, $n = 5$), acute nicotine (AN, $n = 5$), and chronic (CN, $n = 5$).

2.4. Effect of Systemic Administration of Acute and Chronic Nicotine on 5-HT_{2C}R Immunohistochemical Expression in the mPFC, DG, NAc, ST, VTA, SNc, and DRN

The 5-HT_{2C}R was expressed in many brain areas (Tables 1 and 2). The distribution of the 5-HT_{2C}R immunoreactivity was particularly high in the NAc, VTA, CP, and mPFC. These areas contained many immunostained cells distributed throughout their rostro-caudal extension. In the mPFC, 5-HT_{2C}R-IR neurons were located in every layer (Figure 5). In the hippocampal formation, most of the 5-HT_{2C}R-IR neurons were in the granule cells and pyramidal cells layers. However, immunopositive neurons could also be observed in the polymorphic cell layer, in the stratum radiatum, and the stratum oriens (Figure 6). 5-HT_{2C}R immunoreactivity in the habenula-related areas did not show any evident difference comparing the different groups (Tables 1 and 2).

MEDIAL PREFRONTAL CORTEX

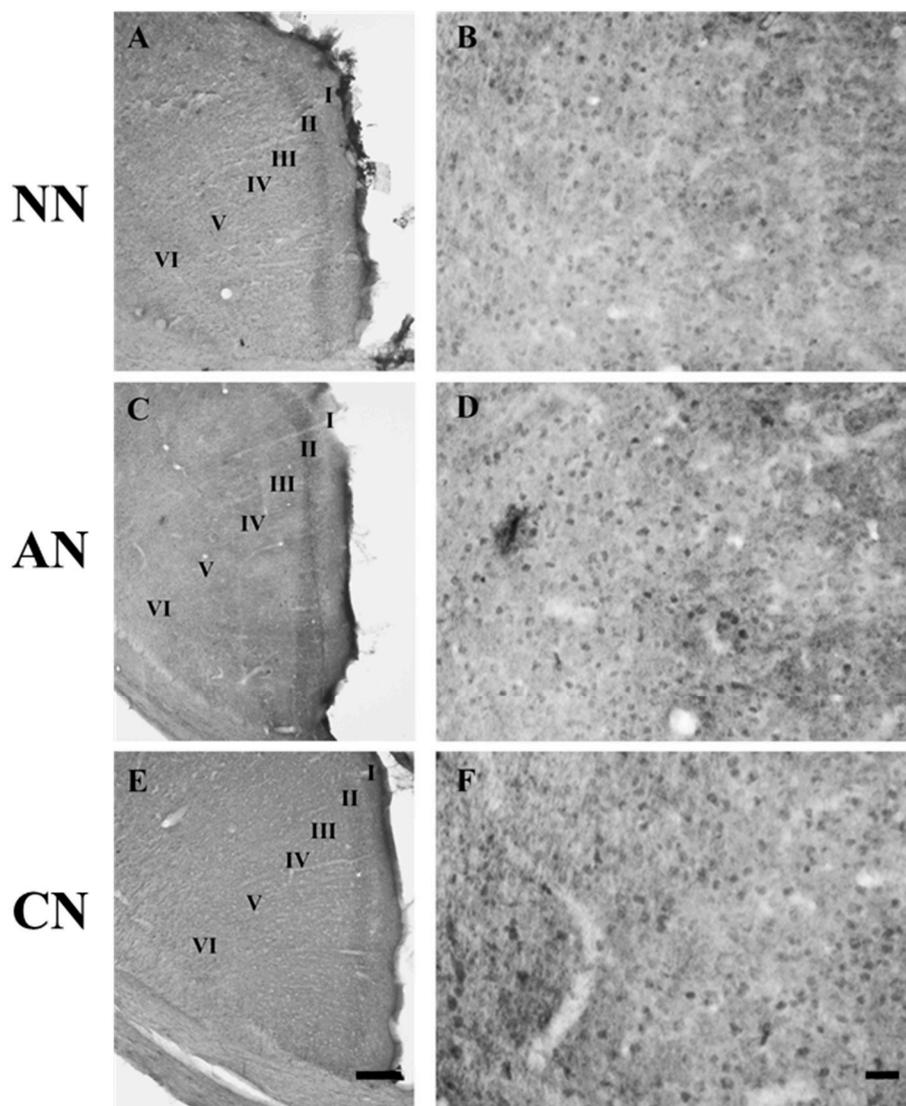


Figure 5. Brightfield photomicrographs of coronal sections showing the distribution of 5-HT_{2C} receptor immunoreactivity in the medial prefrontal cortex (mPFC) of naïve nicotine (NN; (A,B)), acute nicotine (AN; (C,D)) and chronic nicotine (CN; (E,F)) rats. Note that the immunostaining does not show differences comparing the different groups. See text and Tables 1 and 2 for explanations. Scale bar = 200 μ m in (A,C,E) and 60 μ m in (B,D,F). Naïve nicotine (NN, n = 8), acute nicotine (AN, n = 8) and chronic (CN, n = 8).

HIPPOCAMPUS

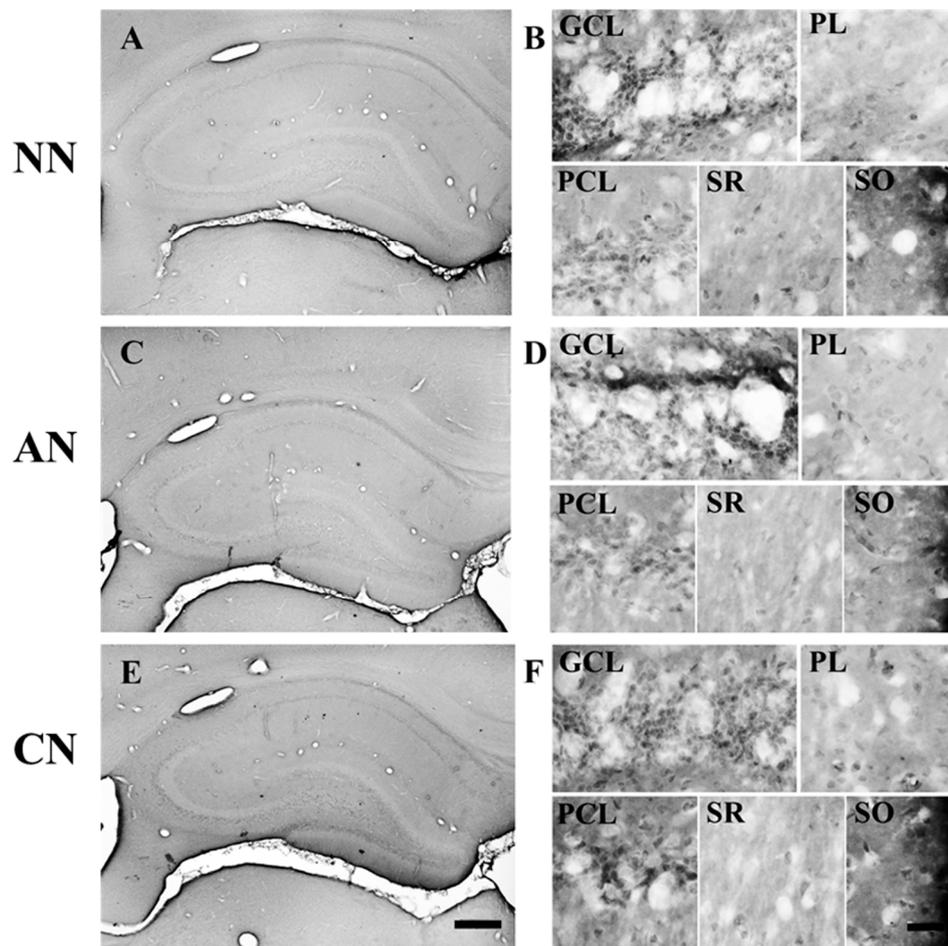


Figure 6. Brightfield photomicrographs of coronal sections showing the distribution of 5-HT_{2C} receptor immunoreactivity in the hippocampal formation of naïve nicotine (NN; (A,B)), acute nicotine (AN; (C,D)) and chronic nicotine (CN; (E,F)) rats. Note that the immunostaining is similar in the different groups. See text and Tables 1 and 2 for explanations. Abbreviations: GCL, granule cell layer of the dentate gyrus; PCL, pyramidal cell layer of the HP proper; PL, polymorphic cell layer of the dentate gyrus; SO, stratum oriens of the HP proper; SR, stratum radiatum of the HP proper. Scale bar = 400 μ m in (A,C,E) and 50 μ m in (B,D,F). Naïve nicotine (NN, n = 8), acute nicotine (AN, n = 8) and chronic (CN, n = 8).

3. Discussion

The main finding of this study is that non-contingent passive exposure both acutely and chronically to low/medium dose of nicotine had a prominent impact on 5-HT_{2C}R signal principally in the LHb of rats while the other brain areas remained mainly unaffected. We observed a complex pattern of 5-HT_{2C}R signal changes both as receptor expression and electrophysiological sensitivity to its pharmacological activation, depending on the time of exposure to nicotine.

Intravenous administration of Ro 60-0175 enhanced the firing rate of LHb neurons in a dose-dependent manner in rats receiving a single acute dose of nicotine. It is noteworthy that the increased neuronal activity concerned 42% of sampled neurons of the LHb while 58% did not respond. The heterogeneity of the responses of LHb neurons to Ro 60-0175 was expected as Ro 60-0175, via the stimulation of 5-HT_{2C}Rs elicited different type of responses including inhibition (24% of neurons), excitation (10%), and no effect (66%) in naïve animals [33]. It appears that short-term exposure to nicotine favors the occurrence

of the excitatory responses triggered by Ro 60-0175 and suppresses the inhibitory ones. Nicotine increases the activity of LHB neurons when acutely administered *in vitro* and *in vivo* [36,43], depolarizing directly LHB neurons via postsynaptic $\alpha 6$ -containing ($\alpha 6^*$) nAChRs but also modulating GABA and GLU input onto LHB neurons [36]. Such an action could change the reactivity to Ro 60-0175 locally, or in regions projecting to the LHB. Alternatively, the short-term exposure to nicotine could be sufficient to alter the distribution of 5-HT_{2C}Rs in the LHB (see below). It is interesting to note that the main electrophysiological response of LHB neurons upon local application of 5-HT_{2C}R agonists *in vitro* and *in vivo* is an excitation [39,40]. It is therefore intriguing that Ro 60-0175's excitatory responses, in addition to the inhibitory responses, were completely lost after the chronic nicotine exposure (6 mg/kg/day for 14 days, *i.p.*).

The inability of the LHB neurons to respond to Ro 60-0175 after chronic nicotine treatment is likely specific to 5-HT_{2C}Rs. In a recent study, we showed that the electrophysiological responses of LHB neurons to the 5-HT_{2A}R agonist TCB-2 were modified only after chronic treatment with nicotine, but the inhibitory ones were slightly amplified [42]. Of note, 5-HT_{2A}Rs and 5-HT_{2C}Rs do not colocalize within the cells of the LHB [33] and distinct outcomes of these two 5-HTRs upon nicotine injection are not surprising. On taking into consideration these different 5-HT_{2A}R signal changes, the low selectivity of Ro 60-0175 toward the 5-HT_{2A}R [41] and the scarce mRNA 5-HT_{2B}R levels in the LHB [44,45], the effects observed after Ro 60-0175 administration in the current study are likely to be attributed to 5-HT_{2C}Rs. Moreover, the loss of response to Ro 60-0175 is also specific to the LHB because the 5-HT_{2C}R agonist is still able to reverse the increase in DA release induced by nicotine in the striatum and the NAc in animals treated repeatedly with nicotine [30,31].

These electrophysiological data prompted us to look at the qualitative expression of 5-HT_{2C}Rs in the LHB after acute and chronic nicotine. One hour from the acute 2 mg/kg nicotine injection, we observed opposite effects on LHB 5-HT_{2C}R signaling with the LHB 5-HT_{2C}R neuropil expression being increased by 30% while the density of LHB 5-HT_{2C}R-IR cells was reduced by about 60%. We confirmed these immunoperoxidase results by double immunofluorescence with the pan-neuronal marker PGP 9.5 [46] that showed that 5-HT_{2C}R-IR LHB cells reduced from 9.3% to 5.1% in acute nicotine treated animals but not by the 5-HT_{2C}R protein expression detected by WB analysis (although an inhibitory trend was observed). On the other hand, chronic nicotine treatment had a strong impact on the 5-HT_{2C}R-IR signal in the LHB. It doubled both the neuropil 5-HT_{2C}R-IR and the LHB 5-HT_{2C}R-IR cell. The quantitative approach using WB confirmed the increased levels of receptor expression in chronic nicotine-treated rats compared to the nicotine-naïve rats.

How the anatomical changes that we observed in the LHB lead to the different desensitization of Ro 60-0175 effects after acute and chronic nicotine exposure, remains a matter of speculation. Considering that 5-HT_{2C}R activation leads generally via G α q/11 activation of phospholipase C (PLC), intracellular calcium (Ca²⁺) mobilization, and final cellular excitation [1,2], the LHB 5-HT_{2C}R plastic changes induced by nicotine likely altered the balance between excitatory and inhibitory (E/I) inputs onto LHB neurons. For example, the net excitatory effect of systemic activation of the 5-HT_{2C}Rs in acute nicotine treatment might be due to an increase of the excitatory 5-HT_{2C}Rs (*i.e.*, on GLU terminals) and a reduction of the expression of the inhibitory postsynaptic 5-HT_{2C}Rs (*i.e.*, on LHB GABAergic interneurons). The lack of effects induced by 5-HT_{2C}R activation after chronic nicotine might be caused instead by a perfect E/I balance via an increase of both presynaptic and postsynaptic receptors. Of note, the responses of 5-HT_{2C}R agonists are critically dependent on the status of dopaminergic transmission. For instance, the lesion of dopaminergic neurons of the substantia nigra changed the electrophysiological response of entopeduncular neurons to local and systemic injection of Ro 60-0175 toward an inhibition [47]. Also, the excitatory effect of Ro 60-0175 on neurons of the shell of the nucleus accumbens was enhanced in slices from methamphetamine-sensitized rats [48]. Alternatively, the 5-HT_{2C}R signal is also finely regulated, with agonists and antagonists both capable of desensitizing receptor signaling and leading to drug tolerance, see [2]. For instance, selective serotonin

reuptake inhibitors that increase 5-HT in the VTA induced desensitization of 5-HT_{2C}Rs as the reduction of the mCPP-induced electrophysiological inhibition of the VTA DA neurons indicates [49]. Therefore, we may hypothesize that nicotine increases the release of 5-HT within LHB [36,44,50] that would induce 5-HT_{2C}R desensitization.

The 5-HT_{2C}R contribution from other areas cannot be excluded. Indeed, even if we did not observe IHC change in the expression of the receptor in other areas, the WB analysis showed an increase in the 5-HT_{2C}R protein expression in the midbrain nuclei after acute nicotine and in the mPFC and HP after chronic nicotine exposure. Little evidence exists on the effect of nicotine on 5-HT_{2C}R expression and in general, the impact is very limited compared to the effect on the other 5-HT₂Rs. In contrast with our results, acute nicotine bitartrate (0.4 mg/kg) after five days of repeated vehicle administration decreased [³H]mesulergine binding to 5-HT_{2C}Rs only in the VTA, nevertheless, these authors did not look at the epithalamic areas. As far as the chronic nicotine effect on 5-HT_{2C}R expression is concerned, the same authors found a decrease in the [³H]mesulergine binding to 5-HT_{2C}Rs of mPFC [8], while instead, we saw an increase of the protein receptor expression. The length of the chronic treatment (5 days vs. our 14 days), and dose (0.4 mg/kg day versus our 6 mg/kg/day) used might explain the different results. On top of that, the difference in techniques (IHC vs quantitative autoradiography) is also an essential factor to take into account, starting with our own data reporting differences between IHC and WB. IHC mainly addresses qualitative changes whereas WB is slightly more quantitative irrespective of the cellular types expressing the receptor. Moreover, as a further confounding factor we had to use different antibodies for IHC and WB analysis (IHC: Santa Cruz mouse monoclonal antibody vs. WB: Abcam rabbit monoclonal antibody).

Combined, the results suggest that the increase in 5-HT_{2C}Rs expression in the mPFC and HP after chronic nicotine is too diffuse to be seen with IHC. Nicotine withdrawal reduced the expression level of [³H]mesulergine binding and decreased 5-HT_{2C}R mRNA editing at the E site in the ventral DG of rats in nicotine-withdrawal, suggesting a shift toward a population of more active 5-HT_{2C}Rs [32]. Considering that the electrophysiological study was carried out only in the LHB we cannot exclude that response to 5-HT_{2C}R activation might be also changed in other areas without a contextual modification of the expression of this receptor but only of their cellular redistribution and/or change in intracellular couplings efficacy and/or oligomerization.

Whatever the study, the data suggest that acute and chronic nicotine induce specific, regional changes of 5-HT_{2C}R function restricted to some brain areas. The behavioral effects inherent to acute and chronic nicotine involve an extended network in which the LHB is crucial in the control of the motivational system [51] and its 5-HT_{2C}R alteration has been involved in the pathogenesis of depressive disorders [40] and drugs of addiction [44,52] including nicotine [42,43]. The 5-HT_{2C}R anti-addictive effects are believed to be based on the inhibition of dopaminergic neurotransmission [2,53] acknowledging that this inhibitory action could be sustained by other brain regions. Indeed, Ro 60-0175 exciting LHB neurons might activate the LHB feed-forward inhibitory control over the VTA neuronal activity via the GABAergic rostromedial tegmental nucleus (RMTg) [54,55]. We showed here that after acute nicotine administration that induces downregulation of LHB 5-HT_{2C}R-IR, Ro 60-0175 produces exclusively excitation of LHB neurons. Consequently, LHB 5-HT_{2C}Rs might contribute to reducing the reward in favor of the aversive nicotine effects by halting the VTA cell firing excitation and contextual elevation of accumbal DA release elicited by acute treatment with nicotine [17,30,31], by enhancing the glutamatergic inputs to the RMTg. Indeed, stimulation of the RMTg neurons projecting to the VTA is known to encode negative but not positive motivational stimuli [56].

The effect of chronic nicotine on the LHB 5-HT_{2C}Rs is more puzzling due to the increase in 5-HT_{2C}R expression associated with the loss of effect of the agonist as discussed above. Yet, the upregulation of the 5-HT_{2C}R expression detected by WB at the level of the mPFC of chronic nicotine-treated animals might contribute to the anti-addiction effect of 5-HT_{2C}R activation. Indeed, infusion of 5-HT_{2C}R agonist MK212 into mPFC reduces

re-instatement of cocaine-seeking [57], and knockdown of mPFC 5-HT_{2C}R resulting in increased motor impulsivity [58].

Moreover, the enhanced 5-HT_{2C}R protein expression in the DG of chronic nicotine treatment could contribute to nicotine-enhanced hippocampal-dependent learning [59] considering the 5-HT_{2C}R memory impairment observed in 5-HT_{2C}R knock out mice [60]. Nevertheless, 5-HT_{2C}R agonist mCPP induced performance deficits in the passive avoidance task in the rat [61] while antagonism at 5-HT_{2C}Rs counteracted rats' retention deficits in a recognition memory task [62], thus suggesting that blockade of the 5-HT_{2C}R may be important for cognition. In any case, less evidence is available on the effect of 5-HT_{2C}R agonists on behaviors related to chronic nicotine exposure. For instance, the chronic co-treatment of nicotine and Ro-60-0175 prevented sensitization to the stimulant effects of chronically administered nicotine [10] but the benefit of 5-HT_{2C}R stimulation could be due to other brain regions including the nucleus accumbens, or the mPFC [48,63]. The effect of chronic nicotine on the LHb neuronal firing is unknown, although we have shown that chronic nicotine treatment induced an effect similar to the LHb lesion on the anxiety-like behavior of rats [37,38]. The loss of LHb feed-forward negative input to the VTA in chronic nicotine-treated may be therefore be important for the establishment and maintenance of nicotine addiction.

This study has some limitations. For instance, the nicotine effects observed here might be specific for the doses used, and therefore the inclusion of different lower and higher doses would be beneficial for the interpretation of the results. A limitation of our findings is that they were obtained in male rats as in many other nicotine animal studies. Sex differences are observed during all stages of nicotine addiction, from initiation to dependence, withdrawal, and relapse [64]. Research including sex differences should be encouraged to develop more effective strategies for smoking cessation.

In conclusion, our data show new insights on 5-HT_{2C}Rs, the LHb and other brain areas in acute and chronic nicotine exposed rats. While these data might support an anti-addictive 5-HT_{2C}R role against acute nicotine, additional data are warranted to further our understanding of the 5-HT_{2C}Rs and the brain circuitry behind the effects of Ro 60-0175 in chronic nicotine exposure. Our findings need to be supported by further studies to establish whether 5-HT_{2C}R agonism may have a therapeutic benefit in nicotine cessation.

4. Materials and Methods

4.1. Animals

Male Sprague-Dawley rats, obtained from Charles River Laboratories in Margate, UK, and maintained at the Department of Physiology and Biochemistry at the University of Malta, were housed at 21 ± 1 °C, with $60 \pm 5\%$ humidity, and a 12 h light/dark cycle (lights on at 7 a.m. and off at 7 p.m.). Food and water were provided ad libitum. Adult rats that weighed 270–320 g on the day of surgery or brain extraction were used. Utmost care was taken to limit the number of rats used and their suffering.

4.2. Drugs

The following compounds were used: (-)-nicotine hydrogen tartrate salt (Sigma Aldrich, St. Louis, MO, USA) and Ro 60-0175 (Tocris Biosciences, UK). Dosage and treatments were based on our previous studies [33,42]. Nicotine was dissolved in saline and pH was adjusted to 7.4 when required. The molecular weight ratio of nicotine hydrogen tartrate to free base nicotine is 2.85. therefore, the dose of nicotine used in this study of 2 mg/kg and 6 mg/kg/day corresponded to 0.7 and 2.10 mg/kg/day, respectively, when expressed as base form.

4.3. Histological Procedures and Immunocytochemistry, Immunoperoxidase Experiments, Double Immunofluorescence Experiments, Specificity of Antibodies, Thionin Staining, Analysis of Sections, Electrophysiological Recordings, and Statistical Analysis

We used an identical anatomical and electrophysiological experimental approach to that of our two recent studies [33,42]. Please refer to Supplementary Materials and [42] for exhaustive details and specific information.

4.3.1. Western Blot Analysis

For Western blot analysis the PFC, HP, LHb, MB, STR, and CR of each rat were dissected, homogenized in cold RIPA buffer (0.3 M NaCl, 0.1% SDS, 25 mM HEPES pH 7.5, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% Triton X-100, 0.5 mM DTT, 0.5% sodium deoxycholate) containing protease inhibitor cocktail (Sigma Aldrich) and stored at -80°C until use.

4.3.2. Western Blotting

Western blotting of 5-HT_{2C}Rs was performed as previously described [65]. Equal amounts of proteins (40 μg) were separated on SDS-PAGE and transferred onto a nitrocellulose membrane (BioRad, Segrate, Italy). After blocking with 5% albumin bovine serum (Sigma Aldrich), membranes were probed with primary antibodies (rabbit monoclonal to 5-HT_{2C}R, Abcam) 1:2000 dilution overnight at 4°C . Protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (GE Healthcare Life Sciences) and the data were evaluated and quantified using ImageJ Free software (NIH, Bethesda, MD, USA). Each experiment was performed at least five times.

Supplementary Materials: Supplementary Materials can be found at <https://www.mdpi.com/article/10.3390/ijms22094775/s1>.

Author Contributions: Conceptualization, C.B., F.D., M.P., and G.D.G.; methodology A.M.G., C.B., G.D.G.; formal analysis, A.G., C.B., F.D., C.T., M.P., and G.D.G.; investigation, A.G., A.M.G., C.B., F.D.; resources, C.B. and G.D.G.; data curation, A.M.G., C.B., F.D., C.T., M.C., P.D.D., M.P., and G.D.G.; writing—original draft preparation, A.M.G., F.D., C.B. and G.D.G.; writing—review and editing, P.D.D., G.D.G.; supervision, C.B. and G.D.G.; project administration, G.D.G.; funding acquisition, G.D.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the University of Malta, and M.P. was supported by a fellowship of the Faculty of Medicine, University of Malta.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and by the Institutional Animal Use and Care Committee (IAUCC) of the University of Malta (15.53 of 22/07/2016).

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

(±)-DOI	2,5-dimethoxy-4-iodoamphetamine
5-HT	5-hydroxytryptamine
5-HT _{2C} Rs	5-HT _{2C} receptors
5-HT _A Rs	5-HT _A receptors
AN	Acute nicotine
CN	Chronic nicotine
DA	Dopamine
DG	dentate gyrus
DRN	dorsal raphe nucleus
ECL	enhanced chemiluminescence
GABA	<i>Gamma</i> -aminobutyric acid
GLU	Glutamate

HT2A	5-HT _A receptor gene
IHC	immunohistochemistry
mPFC	Medial prefrontal cortex
NAc	nucleus accumbens
nAChRs	Nicotinic cholinergic receptors
NN	Naïve nicotine
PBS	phosphate-buffered saline
SNc	substantia nigra pars compacta
ST	Striatum
TCB-2	(4-Bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide
VTA	Ventral tegmental area

References

- Barnes, N.M.; Ahern, G.P.; Becamel, C.; Bockaert, J.; Camilleri, M.; Chaumont-Dubel, S.; Claeysen, S.; Cunningham, K.A.; Fone, K.C.; Gershon, M.; et al. International Union of Basic and Clinical Pharmacology. CX. Classification of Receptors for 5-hydroxytryptamine; Pharmacology and Function. *Pharmacol. Rev.* **2021**, *73*, 310–520. [[CrossRef](#)]
- Di Giovanni, G.; De Deurwaerdere, P. New therapeutic opportunities for 5-HT_{2C} receptor ligands in neuropsychiatric disorders. *Pharmacol. Ther.* **2016**, *157*, 125–162. [[CrossRef](#)]
- Higgins, G.A.; Fletcher, P.J. Therapeutic Potential of 5-HT_{2C} Receptor Agonists for Addictive Disorders. *ACS Chem. Neurosci.* **2015**, *6*, 1071–1088. [[CrossRef](#)]
- Higgins, G.A.; Sellers, E.M.; Fletcher, P.J. From obesity to substance abuse: Therapeutic opportunities for 5-HT_{2C} receptor agonists. *Trends Pharmacol. Sci.* **2013**, *34*, 560–570. [[CrossRef](#)]
- Hayes, D.J.; Mosher, T.M.; Greenshaw, A.J. Differential effects of 5-HT_{2C} receptor activation by WAY 161503 on nicotine-induced place conditioning and locomotor activity in rats. *Behav. Brain Res.* **2009**, *197*, 323–330. [[CrossRef](#)] [[PubMed](#)]
- Quarta, D.; Naylor, C.G.; Stolerman, I.P. The serotonin 2C receptor agonist Ro-60-0175 attenuates effects of nicotine in the five-choice serial reaction time task and in drug discrimination. *Psychopharmacology* **2007**, *193*, 391–402. [[CrossRef](#)] [[PubMed](#)]
- Zaniewska, M.; McCreary, A.C.; Filip, M. Interactions of serotonin (5-HT) 2 receptor-targeting ligands and nicotine: Locomotor activity studies in rats. *Synapse* **2009**, *63*, 653–661. [[CrossRef](#)] [[PubMed](#)]
- Zaniewska, M.; McCreary, A.C.; Wydra, K.; Faron-Gorecka, A.; Filip, M. Context-controlled nicotine-induced changes in the labeling of serotonin (5-HT)_{2A} and 5-HT_{2C} receptors in the rat brain. *Pharm. Rep.* **2015**, *67*, 451–459. [[CrossRef](#)] [[PubMed](#)]
- Fletcher, P.J.; Rizos, Z.; Noble, K.; Soko, A.D.; Silenieks, L.B.; Lê, A.D.; Higgins, G.A. Effects of the 5-HT_{2C} receptor agonist Ro60-0175 and the 5-HT_{2A} receptor antagonist M100907 on nicotine self-administration and reinstatement. *Neuropharmacology* **2012**, *62*, 2288–2298. [[CrossRef](#)] [[PubMed](#)]
- Grottick, A.; Corrigan, W.A.; Higgins, G.A. Activation of 5-HT 2C receptors reduces the locomotor and rewarding effects of nicotine. *Psychopharmacology* **2001**, *157*, 292–298. [[CrossRef](#)] [[PubMed](#)]
- Levin, E.D.; Johnson, J.E.; Slade, S.; Wells, C.; Cauley, M.; Petro, A.; Rose, J.E. Lorcaserin, a 5-HT_{2C} Agonist, Decreases Nicotine Self-Administration in Female Rats. *J. Pharmacol. Exp. Ther.* **2011**, *338*, 890–896. [[CrossRef](#)] [[PubMed](#)]
- Harvey-Lewis, C.; Li, Z.; Higgins, G.A.; Fletcher, P.J. The 5-HT_{2C} receptor agonist lorcaserin reduces cocaine self-administration, reinstatement of cocaine-seeking and cocaine induced locomotor activity. *Neuropharmacology* **2016**, *101*, 237–245. [[CrossRef](#)] [[PubMed](#)]
- Price, A.E.; Anastasio, N.C.; Stutz, S.J.; Hommel, J.D.; Cunningham, K.A. Serotonin 5-HT_{2C} Receptor Activation Suppresses Binge Intake and the Reinforcing and Motivational Properties of High-Fat Food. *Front. Pharmacol.* **2018**, *9*. [[CrossRef](#)] [[PubMed](#)]
- Shanahan, W.R.; Rose, J.E.; Glicklich, A.; Stubbe, S.; Sanchez-Kam, M. Lorcaserin for Smoking Cessation and Associated Weight Gain: A Randomized 12-Week Clinical Trial. *Nicotine Tob. Res.* **2017**, *19*, 944–951. [[CrossRef](#)]
- Rose, J.E.; Davis, J.M. Combination Lorcaserin and Nicotine Patch for Smoking Cessation without Weight Gain. *Nicotine Tob. Res.* **2020**, *22*, 1627–1631. [[CrossRef](#)] [[PubMed](#)]
- Sharretts, J.; Galescu, O.; Gomatam, S.; Andraca-Carrera, E.; Hampp, C.; Yanoff, L. Cancer Risk Associated with Lorcaserin—The FDA’s Review of the CAMELLIA-TIMI 61 Trial. *N. Engl. J. Med.* **2020**, *383*, 1000–1002. [[CrossRef](#)] [[PubMed](#)]
- Di Matteo, V.; Pierucci, M.; Di Giovanni, G.; Benigno, A.; Esposito, E. The neurobiological bases for the pharmacotherapy of nicotine addiction. *Curr. Pharm. Des.* **2007**, *13*, 1269–1284. [[CrossRef](#)] [[PubMed](#)]
- Di Matteo, V.; Pierucci, M.; Benigno, A.; Esposito, E.; Crescimanno, G.; Di Giovanni, G. Critical role of nitric oxide on nicotine-induced hyperactivation of dopaminergic nigrostriatal system: Electrophysiological and neurochemical evidence in rats. *CNS Neurosci. Ther.* **2010**, *16*, 127–136. [[CrossRef](#)]
- Picciotto, M.R.; Corrigan, W.A. Neuronal Systems Underlying Behaviors Related to Nicotine Addiction: Neural Circuits and Molecular Genetics. *J. Neurosci.* **2002**, *22*, 3338–3341. [[CrossRef](#)]
- Di Chiara, G.; Bassareo, V. Reward system and addiction: What dopamine does and doesn’t do. *Curr. Opin. Pharmacol.* **2007**, *7*, 69–76. [[CrossRef](#)]
- Di Mascio, M.; Di Giovanni, G.; Di Matteo, V.; Esposito, E. Decreased chaos of midbrain dopaminergic neurons after serotonin denervation. *Neuroscience* **1999**, *92*, 237–243. [[CrossRef](#)]

22. De Deurwaerdere, P.; Di Giovanni, G. Serotonergic modulation of the activity of mesencephalic dopaminergic systems: Therapeutic implications. *Prog. Neurobiol.* **2017**, *151*, 175–236. [[CrossRef](#)]
23. Di Giovanni, G.; De Deurwaerdere, P.; Di Mascio, M.; Di Matteo, V.; Esposito, E.; Spampinato, U. Selective blockade of serotonin-2C/2B receptors enhances mesolimbic and mesostriatal dopaminergic function: A combined in vivo electrophysiological and microdialysis study. *Neuroscience* **1999**, *91*, 587–597. [[CrossRef](#)]
24. De Deurwaerdere, P.; Ramos, M.; Bharatiya, R.; Puginier, E.; Chagraoui, A.; Manem, J.; Cuboni, E.; Pierucci, M.; Deidda, G.; Casarrubea, M.; et al. Lorcaserin bidirectionally regulates dopaminergic function site-dependently and disrupts dopamine brain area correlations in rats. *Neuropharmacology* **2020**, *166*, 107915. [[CrossRef](#)] [[PubMed](#)]
25. De Deurwaerdere, P.; Navailles, S.; Berg, K.A.; Clarke, W.P.; Spampinato, U. Constitutive activity of the serotonin2C receptor inhibits in vivo dopamine release in the rat striatum and nucleus accumbens. *J. Neurosci.* **2004**, *24*, 3235–3241. [[CrossRef](#)] [[PubMed](#)]
26. Di Giovanni, G.; Di Matteo, V.; La Grutta, V.; Esposito, E. m-Chlorophenylpiperazine excites non-dopaminergic neurons in the rat substantia nigra and ventral tegmental area by activating serotonin-2C receptors. *Neuroscience* **2001**, *103*, 111–116. [[CrossRef](#)]
27. Di Matteo, V.; Di Giovanni, G.; Di Mascio, M.; Esposito, E. Biochemical and electrophysiological evidence that RO 60-0175 inhibits mesolimbic dopaminergic function through serotonin2C receptors. *Brain Res.* **2000**, *865*, 85–90. [[CrossRef](#)]
28. Porras, G.; Di Matteo, V.; Fracasso, C.; Lucas, G.; De Deurwaerdere, P.; Caccia, S.; Esposito, E.; Spampinato, U. 5-HT 2A and 5-HT 2C/2B receptor subtypes modulate dopamine release induced in vivo by amphetamine and morphine in both the rat nucleus accumbens and striatum. *Neuropsychopharmacology* **2002**, *26*, 311–324. [[CrossRef](#)]
29. Willins, D.L.; Meltzer, H.Y. Serotonin 5-HT2C agonists selectively inhibit morphine-induced dopamine efflux in the nucleus accumbens. *Brain Res.* **1998**, *781*, 291–299. [[CrossRef](#)]
30. Di Matteo, V.; Pierucci, M.; Esposito, E. Selective stimulation of serotonin2c receptors blocks the enhancement of striatal and accumbal dopamine release induced by nicotine administration. *J. Neurochem.* **2004**, *89*, 418–429. [[CrossRef](#)] [[PubMed](#)]
31. Pierucci, M.; Di Matteo, V.; Esposito, E. Stimulation of serotonin2C receptors blocks the hyperactivation of midbrain dopamine neurons induced by nicotine administration. *J. Pharmacol. Exp. Ther.* **2004**, *309*, 109–118. [[CrossRef](#)] [[PubMed](#)]
32. Zaniowska, M.; Alenina, N.; Wydra, K.; Fröhler, S.; Kuśmider, M.; McCreary, A.C.; Chen, W.; Bader, M.; Filip, M. Discovering the mechanisms underlying serotonin (5-HT)2A and 5-HT2C receptor regulation following nicotine withdrawal in rats. *J. Neurochem.* **2015**, *134*, 704–716. [[CrossRef](#)] [[PubMed](#)]
33. Delicata, F.; Bombardi, C.; Pierucci, M.; Di Maio, R.; De Deurwaerdere, P.; Di Giovanni, G. Preferential modulation of the lateral habenula activity by serotonin-2A rather than-2C receptors: Electrophysiological and neuroanatomical evidence. *CNS Neurosci. Ther.* **2018**, *24*, 721–733. [[CrossRef](#)] [[PubMed](#)]
34. Hikosaka, O. The habenula: From stress evasion to value-based decision-making. *Nat. Rev. Neurosci.* **2010**, *11*, 503–513. [[CrossRef](#)]
35. Pierucci, M.; Chambers, S.; Partridge, L.; De Deurwaerdere, P.; Di Giovanni, G. Role of central serotonin receptors in nicotine addiction. In *Nicotinic Receptors*; Humana Press: New York, NY, USA, 2014; pp. 279–305.
36. Zuo, W.; Xiao, C.; Gao, M.; Hopf, F.W.; Krnjevic, K.; McIntosh, J.M.; Fu, R.; Wu, J.; Bekker, A.; Ye, J.H. Nicotine regulates activity of lateral habenula neurons via presynaptic and postsynaptic mechanisms. *Sci. Rep.* **2016**, *6*, 32937. [[CrossRef](#)]
37. Casarrubea, M.; Davies, C.; Faulisi, F.; Pierucci, M.; Colangeli, R.; Partridge, L.; Chambers, S.; Cassar, D.; Valentino, M.; Muscat, R. Acute nicotine induces anxiety and disrupts temporal pattern organization of rat exploratory behavior in hole-board: A potential role for the lateral habenula. *Front. Cell. Neurosci.* **2015**, *9*, 197. [[CrossRef](#)]
38. Casarrubea, M.; Davies, C.; Pierucci, M.; Colangeli, R.; Deidda, G.; Santangelo, A.; Aiello, S.; Crescimanno, G.; Di Giovanni, G. The impact of chronic daily nicotine exposure and its overnight withdrawal on the structure of anxiety-related behaviors in rats: Role of the lateral habenula. *Prog. Neuro-Psychopharmacol. Biol. Psychiatry* **2021**, *105*, 110131. [[CrossRef](#)]
39. Zuo, W.; Zhang, Y.; Xie, G.; Gregor, D.; Bekker, A.; Ye, J.H. Serotonin stimulates lateral habenula via activation of the post-synaptic serotonin 2/3 receptors and transient receptor potential channels. *Neuropharmacology* **2016**, *101*, 449–459. [[CrossRef](#)]
40. Han, L.N.; Zhang, L.; Li, L.B.; Sun, Y.N.; Wang, Y.; Chen, L.; Guo, Y.; Zhang, Y.M.; Zhang, Q.J.; Liu, J. Activation of serotonin(2C) receptors in the lateral habenular nucleus increases the expression of depression-related behaviors in the hemiparkinsonian rat. *Neuropharmacology* **2015**, *93*, 68–79. [[CrossRef](#)]
41. Martin, J.R.; Bos, M.; Jenck, F.; Moreau, J.; Mutel, V.; Sleight, A.J.; Wichmann, J.; Andrews, J.S.; Berendsen, H.H.; Broekkamp, C.L.; et al. 5-HT2C receptor agonists: Pharmacological characteristics and therapeutic potential. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 913–924.
42. Bombardi, C.; Delicata, F.; Tagliavia, C.; Pierucci, M.; Deidda, G.; Casarrubea, M.; De Deurwaerdere, P.; Di Giovanni, G. Acute and Chronic Nicotine Exposures Differentially Affect Central Serotonin 2A Receptor Function: Focus on the Lateral Habenula. *Int. J. Mol. Sci.* **2020**, *21*, 1873. [[CrossRef](#)] [[PubMed](#)]
43. Pierucci, M.; Pitruzzella, A.; Valentino, M.; Zammit, C.; Muscat, R.; Benigno, A.; Di Giovanni, G. Lateral Habenula contribution in nicotine addiction: Focus on dopamine, GABA and serotonin interactions. *Malta Med. J.* **2011**, *23*, 32–37.
44. Zuo, W.; Wu, L.; Mei, Q.; Zuo, Q.; Zhou, Z.; Fu, R.; Li, W.; Wu, W.; Matthew, L.; Ye, J.-H. Adaptation in 5-HT2 receptors-CaMKII signaling in lateral habenula underlies increased nociceptive-sensitivity in ethanol-withdrawn rats. *Neuropharmacology* **2019**, *158*, 107747. [[CrossRef](#)] [[PubMed](#)]
45. Aizawa, H.; Kobayashi, M.; Tanaka, S.; Fukai, T.; Okamoto, H. Molecular characterization of the subnuclei in rat habenula. *J. Comp. Neurol.* **2012**, *520*, 4051–4066. [[CrossRef](#)] [[PubMed](#)]

46. Thompson, R.; Doran, J.; Jackson, P.; Dhillon, A.; Rode, J. PGP 9.5—A new marker for vertebrate neurons and neuroendocrine cells. *Brain Res.* **1983**, *278*, 224–228. [[CrossRef](#)]
47. Lagièrre, M.; Navailles, S.; Mignon, L.; Roumegous, A.; Chesselet, M.F.; De Deurwaerdère, P. The enhanced oral response to the 5-HT₂ agonist Ro 60-0175 in parkinsonian rats involves the entopeduncular nucleus: Electrophysiological correlates. *Exp. Brain Res.* **2013**, *230*, 513–524. [[CrossRef](#)]
48. Graves, S.M.; Clark, M.J.; Traynor, J.R.; Hu, X.-T.; Napier, T.C. Nucleus accumbens shell excitability is decreased by methamphetamine self-administration and increased by 5-HT_{2C} receptor inverse agonism and agonism. *Neuropharmacology* **2015**, *89*, 113–121. [[CrossRef](#)]
49. Prisco, S.; Esposito, E. Differential effects of acute and chronic fluoxetine administration on the spontaneous activity of dopaminergic neurones in the ventral tegmental area. *Br. J. Pharmacol.* **1995**, *116*, 1923–1931. [[CrossRef](#)]
50. Tchenio, A.; Valentinova, K.; Mameli, M. Can the Lateral Habenula Crack the Serotonin Code? *Front. Synaptic Neurosci.* **2016**, *8*, 34. [[CrossRef](#)]
51. Graziane, N.M.; Neumann, P.A.; Dong, Y. A focus on reward prediction and the lateral habenula: Functional alterations and the behavioral outcomes induced by drugs of abuse. *Front. Synaptic Neurosci.* **2018**, *10*, 12. [[CrossRef](#)]
52. Fu, R.; Mei, Q.; Shiwalkar, N.; Zuo, W.; Zhang, H.; Gregor, D.; Patel, S.; Ye, J.-H. Anxiety during alcohol withdrawal involves 5-HT_{2C} receptors and M-channels in the lateral habenula. *Neuropharmacology* **2020**, *163*, 107863. [[CrossRef](#)]
53. Higgins, G.A.; Sellers, E.M. 5-HT_{2A} and 5-HT_{2C} receptors as potential targets for the treatment of nicotine use and dependence. *Prog. Brain Res.* **2021**, *259*, 229–263. [[CrossRef](#)]
54. Kaufling, J.; Veinante, P.; Pawlowski, S.A.; Freund-Mercier, M.-J.; Barrot, M. Afferents to the GABAergic tail of the ventral tegmental area in the rat. *J. Comp. Neurol.* **2009**, *513*, 597–621. [[CrossRef](#)]
55. Lecca, S.; Melis, M.; Luchicchi, A.; Ennas, M.G.; Castelli, M.P.; Muntoni, A.L.; Pistis, M. Effects of drugs of abuse on putative rostromedial tegmental neurons, inhibitory afferents to midbrain dopamine cells. *Neuropsychopharmacology* **2011**, *36*, 589–602. [[CrossRef](#)]
56. Li, H.; Vento, P.J.; Parrilla-Carrero, J.; Pullmann, D.; Chao, Y.S.; Eid, M.; Jhou, T.C. Three rostromedial tegmental afferents drive triply dissociable aspects of punishment learning and aversive valence encoding. *Neuron* **2019**, *104*, 987–999. [[CrossRef](#)]
57. Pentkowski, N.S.; Duke, F.D.; Weber, S.M.; Pockros, L.A.; Teer, A.P.; Hamilton, E.C.; Thiel, K.J.; Neisewander, J.L. Stimulation of Medial Prefrontal Cortex Serotonin 2C (5-HT_{2C}) Receptors Attenuates Cocaine-Seeking Behavior. *Neuropsychopharmacology* **2010**, *35*, 2037–2048. [[CrossRef](#)] [[PubMed](#)]
58. Anastasio, N.C.; Stutz, S.J.; Fink, L.H.; Swinford-Jackson, S.E.; Sears, R.M.; DiLeone, R.J.; Rice, K.C.; Moeller, F.G.; Cunningham, K.A. Serotonin (5-HT) 5-HT_{2A} Receptor (5-HT_{2AR}):5-HT_{2CR} Imbalance in Medial Prefrontal Cortex Associates with Motor Impulsivity. *ACS Chem. Neurosci.* **2015**, *6*, 1248–1258. [[CrossRef](#)] [[PubMed](#)]
59. Kutlu, M.G.; Gould, T.J. Nicotinic modulation of hippocampal cell signaling and associated effects on learning and memory. *Physiol. Behav.* **2016**, *155*, 162–171. [[CrossRef](#)] [[PubMed](#)]
60. Tecott, L.H.; Logue, S.F.; Wehner, J.M.; Kauer, J.A. Perturbed dentate gyrus function in serotonin 5-HT_{2C} receptor mutant mice. *Proc. Natl. Acad. Sci.* **1998**, *95*, 15026–15031. [[CrossRef](#)]
61. Misane, I.; Ögren, S.O. Multiple 5-HT receptors in passive avoidance: Comparative studies of p-chloroamphetamine and 8-OH-DPAT. *Neuropsychopharmacology* **2000**, *22*, 168–190. [[CrossRef](#)]
62. Pitsikas, N.; Sakellaridis, N. The 5-HT_{2C} receptor antagonist RO 60-0491 counteracts rats' retention deficits in a recognition memory task. *Brain Res.* **2005**, *1054*, 200–202. [[CrossRef](#)] [[PubMed](#)]
63. Howell, L.L.; Cunningham, K.A. Serotonin 5-HT₂ receptor interactions with dopamine function: Implications for therapeutics in cocaine use disorder. *Pharmacol. Rev.* **2015**, *67*, 176–197. [[CrossRef](#)] [[PubMed](#)]
64. Pogun, S.; Yazarbas, G.; Nesil, T.; Kanit, L. Sex differences in nicotine preference. *J. Neurosci. Res.* **2017**, *95*, 148–162. [[CrossRef](#)] [[PubMed](#)]
65. Orban, G.; Bombardi, C.; Marino Gammazza, A.; Colangeli, R.; Pierucci, M.; Pomara, C.; Pessia, M.; Bucchieri, F.; Benigno, A.; Smolders, I.; et al. Role(s) of the 5-HT_{2C} receptor in the development of maximal dentate activation in the hippocampus of anesthetized rats. *CNS Neurosci. Ther.* **2014**, *20*, 651–661. [[CrossRef](#)]