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Neurogenic Potential Assessment and Pharmacological Characterization of 6-Methoxy-1,2,3,4-Tetrahydro-β-Carboline (Pinoline) and Melatonin – Pinoline Hybrids

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Dedicated to our dear colleague Prof. José Elguero on the occasion of his 80th birthday

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ABSTRACT

6-Methoxy-1,2,3,4-tetrahydro-β-carboline (pinoline) and *N*-acetyl-5-methoxytryptamine (melatonin) are both structurally related to 5-hydroxytryptamine (serotonin). Here we describe the design, synthesis and characterization of a series of melatonin rigid analogues resulting from the hybridization of both pinoline and melatonin structures. The pharmacological evaluation of melatonin – pinoline hybrids comprises serotonergic and melatonergic receptors, metabolic enzymes (monoamine oxidases), antioxidant potential, the *in vitro* blood-brain barrier permeability, and neurogenic studies. Pinoline at trace concentrations and 2-acetyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline (**2**) were able to stimulate early neurogenesis and neuronal maturation in an *in vitro* model of neural stem cells isolated from the adult rat subventricular zone. Such effects are presumably mediated via serotonergic and melatonergic stimulation respectively.

KEYWORDS: Melatonin, pinoline, neurogenesis, serotonergic receptors, melatonergic receptors, monoamine oxidases, antioxidants

Introduction

Pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline, sometimes also designated as 5-methoxytryptoline) belongs to a vast family of bioactive pyridoindoles, found in foods, plants and animals.[1] Pinoline and melatonin are both structurally related to serotonin (5-hydroxytryptamine, 5-HT) and to its methoxyl derivative (5-methoxytryptamine, 5-MeOT). Regarding 5-MeOT, pinoline is a conformational-restricted analogue and melatonin, its acetamide (Figure 1).

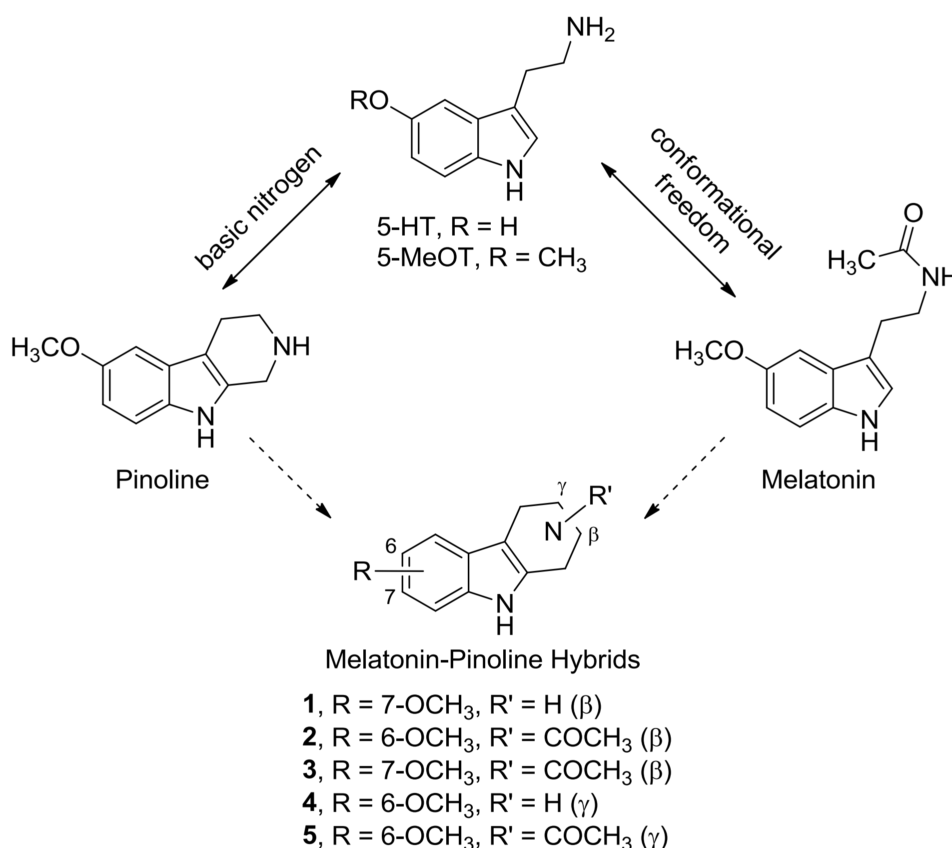


Figure 1. Schematic representation of the approach used in this work and its parent structures. Filled arrows represent the common features of the depicted structures. Broken arrows show the hybridization of pinoline and melatonin.

Initially, pinoline was proposed as the endogenous ligand of the [^{3}H]imipramine binding site and the adrenoglomerulotropin factor, responsible for the pineal regulation of the adrenal gland.[2-5] Numerous efforts were done to determine the pinoline levels in different tissues, resulting in a wide variety of concentrations that ranged from few ng/g up to 1 µg/g in brain, pineal and adrenal gland.[6] However, criticism may apply in certain early determinations since the analytical procedures require a fine design of the protocol to avoid artifact formation.[7] Langer et al. (1985) reported the concentration of pinoline in the pineal gland to be as low as 2 ng/g in rats,[8] whereas more recently Barker et al. (2013) could not detect pinoline in samples of the same tissue.[9] As the synthesis of pinoline by pineal gland appears to be inexistent, at most scant, such levels question the relevance of the neuroendocrine production of pinoline. Even if there is no support for an active role of pineal gland in the biosynthesis of pinoline, there are reports in the literature suggesting a plausible spontaneous non-enzymatic generation of β-carbolines from their corresponding tryptamine precursor.[10-13] In the case of pinoline, its precursor 5-MeOT is indeed synthesized in the pineal gland.[14]

Numerous reports on the pharmacology of pinoline can be found in the scientific literature before controversy arouse on its occurrence as an endogenous neurochemical of the pineal gland.[3, 6] The current interest of this molecule is focused on the study of its antioxidant properties and as a probe for cytochrome activity.[15-18] In recent years and beyond its scientific interest, pinoline has also attracted attention of new-age religious groups that ascribe enlightening properties to this molecule, probably due to its structural resemblance to *N*,*N*-dimethyltryptamine, nevertheless no reports of its administration to humans have ever been published in peer-reviewed publications.[18, 19] The β-carboline scaffold that configures the tricyclic structure of pinoline is also present in alkaloids found in different plant species like *Banisteriopsis caapi* (Malpighiaceae),

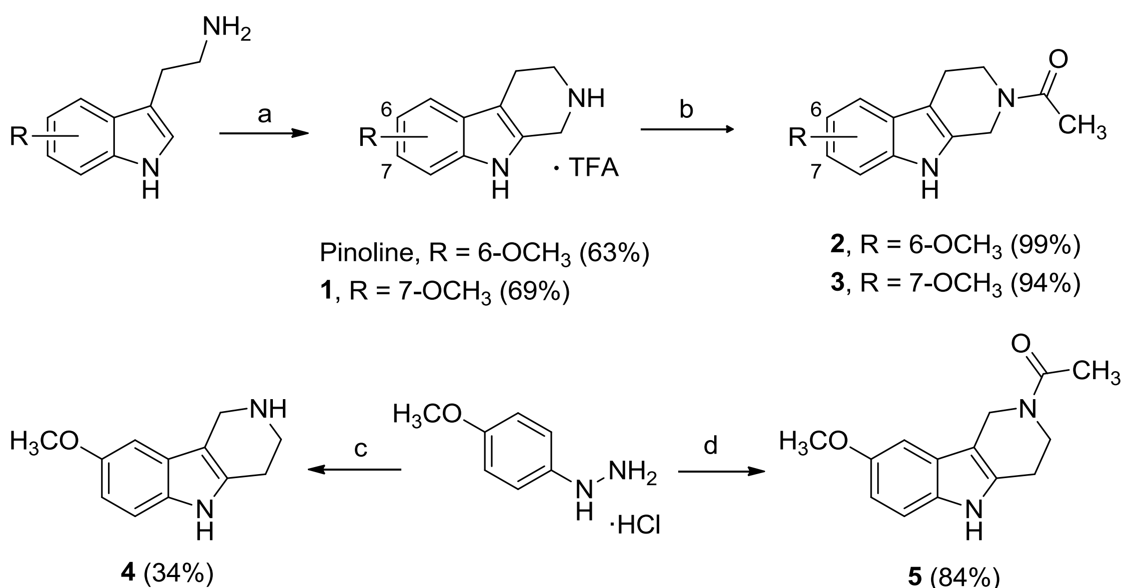
employed in Amazonian shamanism for the preparation of the hallucinogenic beverage Ayahuasca.[20-22] Some of these alkaloids are reported to be hallucinogenic *per se* or acting as a coadjutants by inhibiting monoaminoxidase A (MAO-A).[19, 22, 23]

Pinoline shares with melatonin the 5-methoxyindole, although two major structural differences exist between them: the nature of the non-aromatic nitrogen and the number of free-rotatable bonds. Like serotonin, pinoline is a secondary amine, whose basicity suggests a likely protonated ammonium state at physiologic pH with its corresponding positive charge, and a plausible neurotransmitter-like pharmacology. Melatonin is a primary amide and the endogenous ligand of melatonergic receptors MT_1 and MT_2.[24] Its carbamoyl moiety is able to intervene both as a donor and acceptor in hydrogen bonds, but lacks any absolute charge. With regard on their flexibility, melatonin and serotonin have an ethylene chain that can adopt several energetically equivalent conformations by free-rotation around the sp^3 carbons. Conversely, the additional methylene that configures the tetrahydropyrido ring of pinoline limits the conformational freedom of the molecule, being the relative spatial disposition rather fixed. Based on these observations and in our interest in melatonin-based potential drugs,[25-28] we wished to develop a series of melatonin-restricted analogues by integrating the main structural features of melatonin within the carboline scaffold of pinoline, thus hybridizing both structures (Figure 1). The synthesized compounds **1-5** were pharmacologically characterized and compared to their parent and related structures in serotonergic and melatonergic receptors, metabolic enzymes (monoamine oxidases), and in two models related to assess their antioxidant potential and blood-brain barrier permeability. Provided the neurogenic potential of melatonin[29, 30] and our interest in developing new potential brain-repairing agents,[25] we also explored the

neurogenic potential of the orthodox melatonin – pinoline hybrid **2** and the parent compound pinoline, in neural stem cells.

RESULTS

Pinoline and related 1,2,3,4-tetrahydro-β-carbolines **1-3** were obtained as outlined in Scheme 1. Following an acid-catalysed Pictet-Spengler procedure,[31] the treatment of the appropriate tryptamine with paraformaldehyde in the presence of trifluoroacetic acid (TFA) afforded pinoline and its 7-methoxy counterpart (**1**), both as trifluoroacetate salts in fair yields. Acetylation of the above free bases with acetic anhydride proceeded smoothly, affording acetylated tetrahydro-β-carbolines **2** and **3** in excellent yields.



Scheme 1. Synthesis of pinoline and related 1,2,3,4-tetrahydro-(β or γ)-carbolines **1-5**. *Reagents and conditions*. (a) paraformaldehyde, TFA, DCM, rt, 72h; (b) acetic anhydride, TEA, MeCN, rt, 1h; (c) 4-piperidone, EtOH, conc. HCl, reflux, 3h; (d) 1-acetyl-4-piperidinone, methylurea:tartartic acid (70:30), 80 ºC, 90 min.

1,2,3,4-Tetrahydro-γ-carbolines **4** and **5** were obtained via Fischer indole synthesis, starting from (4-methoxyphenyl)hydrazine hydrochloride and the corresponding 4-piperidone using two different procedures (Scheme 1). In the case of **4** a traditional acid-catalysed method was employed: the starting hydrazine hydrochloride was refluxed with 4-piperidone in a mixture of ethanol and aq. HCl, obtaining **4** in low yield.[32] In the synthesis of **5** a different acid source was used. Deep eutectic mixtures have proven to be cheap and versatile solvents in organic chemistry and biocatalysis with similar properties to traditional ionic liquids, with the additional advantage of being green media of much less toxicity to environment and humans.[33, 34] In this case, a eutectic mixture of methylurea and tartaric acid (70:30) provided an acidic polar medium able to catalyse Fischer indole synthesis,[35] and thus **5** was obtained from (4-methoxyphenyl)hydrazine and 1-acetyl-4-piperidinone in very good yield.

The binding affinities of pinoline and melatonin – pinoline hybrids (**1**-**5**) for different serotonin receptors and transporter (5-HTR and SERT, respectively) were determined in radioligand displacement studies in transfected cells, stably or transiently expressing human 5-HTR or SERT. The results are summarized in Table 1.

Table 1. Binding profiles to human 5-HTR, and SERT determined by radioligand displacement. K_i, μM ($pK_i \pm$ SEM).[a]

Compd.	5-HT_{1A}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	5-HT_{7}	SERT
pinoline	4.335 (5.36 ± 0.07)	2.210 (5.66 ± 0.09)	0.156 (6.81 ± 0.06)	1.503 (5.82 ± 0.06)	0.607 (6.22 ± 0.06)	0.172 (6.76 ± 0.05)
1	5.797 (5.24 ± 0.08)	2.919 (5.53 ± 0.08)	0.385 (6.41 ± 0.05)	1.258 (5.90 ± 0.07)	1.906 (5.72 ± 0.07)	0.614 (6.21 ± 0.09)
4	>10	0.229 (6.64 ± 0.07)	0.194 (6.71 ± 0.08)	0.219 (6.66 ± 0.06)	0.938 (6.03 ± 0.06)	>10

[a] Data are the mean ± SEM of 3 independent experiments.

Only pinoline and compounds **1** and **4**, bearing a protonatable amino group, showed affinity for 5-HTR, whereas their acetylated counterparts were inactive (data not shown). All three protonatable tetrahydrocarbolines showed rather similar binding profiles: K_i values in the low- or sub-micromolar range for 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} and 5-HT_7 receptors. The similarities were even more obvious in the β-carboline derivatives, pinoline, and **1**, in which the relative position of the methoxy group did not appear to be relevant except for 5-HT_7R. The relative position of the protonatable nitrogen seemed to be more relevant when comparing the γ-carboline **4** and pinoline. The nitrogen in γ-position abolished the binding to 5-HT_{1A}R receptors, but increased the affinity for 5-HT_{2A}R and 5-HT_{2C}R while maintaining the affinity for 5-HT_{2B}R and 5-HT_7R unchanged. In the case of the SERT the same structure-activity relationship (SAR) was found, the affinity of the methoxy-regioisomers pinoline and **1** was nearly identical, whereas the γ-carboline **4** lacked a significant affinity for this transporter.

The intrinsic activity of pinoline, **1** and **4** was further determined at 5-HT_2 receptors (Table 2). The β-carboline derivatives (pinoline and **1**) showed a very similar qualitative pharmacological profile: partial agonism at the 5-HT_{2A}R, antagonism at the 5-HT_{2B}R (both in the low micromolar range) and full agonism at the 5-HT_{2C}R (sub-micromolar range). Pinoline was a fairly potent agonist at 5-HT_{2C}R whereas γ-carboline **4** was unable to activate any of the tested subtypes.

Table 2. Functional characterization of non-acetylated compounds at 5-HT_2R determined as intracellular calcium mobilization.[a]

	5-HT_{2A}R			5-HT_{2B}R		5-HT_{2C}R		
Compd.	EC_{50}	E_{max}	n	IC_{50}	n	EC_{50}	E_{max}	n
pinoline	2.14 ± 0.10	75	1.58	1.12 ± 0.05	1.5	0.033 ± 0.001	95	1.9
1	3.28 ± 0.15	57	1.35	3.63 ± 0.17	1.3	0.120 ± 0.005	100	1.7
4	–[b]			19.8 ± 0.9	1.4	–[b]		

[a]All EC_{50} or IC_{50} values in μM with ± SEM values. E_{max} is expressed as a percentage of the maximum response elicited by 5-HTR. n represents the Hill coefficient. Experiments were run in triplicate. [b]γ-Carboline **4** was unable to activate 5-HT_{2A}R nor 5-HT_{2C}R receptors.

The ability of pinoline and melatonin – pinoline hybrids **1-5** to inhibit monoamine oxidase A (MAO-A) and B (MAO-B) was assessed. All compounds were inactive at MAO-B and only basic tetrahydro-β-carbolines, namely pinoline and derivative **1**, showed significant inhibition of the MAO-A isoform, being the inhibition elicited by pinoline (IC_{50} = 41.5 ± 6.3 μM) ~25-fold lower than that of **1** (IC_{50} = 1.3 ± 0.3 μM).

The affinity of pinoline and hybrids **1**-**5** for melatonin receptors was determined by displacement of the radiolabelled ligand [^{125}I]iodomelatonin. Out of the six compounds tested only **2** showed significant binding activity for the melatonin receptors [K_i (MT_1) = 0.69 ± 0.03 μM; K_i (MT_2) = 0.33 ± 0.01 μM), being the affinity for MT_2 two-fold higher than for MT_1. None of its position isomers (**5** or **3**) showed any significant activity. Neither did any of the parent amino compounds, pinoline or its isomers (**1** and **4**).

Compound **2** was functionally characterized at MT_1 and MT_2 receptor subtypes in the [^{35}S]GTPγS binding assay. It is a partial agonist at both receptor subtypes [E_{max} (MT_1) = 70%; E_{max} (MT_2) = 77%] in the sub-micromolar range [EC_{50} (MT_1) = 0.34 ± 0.08 μM; EC_{50} (MT_2) = 0.14 ± 0.07 μM], being slightly more potent at the MT_2 receptor.

According to the brain-blood barrier model PAMPA (parallel artificial membrane permeability assay), pinoline and all melatonin – pinoline hybrids (**1**-**5**) showed positive CNS-permeability (see Supporting Information). In the case of pinoline, these results are in agreement with previous reports in which radiolabelled pinoline demonstrated its ability to reach the central nervous system.[36, 37]

Table 3 shows the antioxidant properties of all compounds determined in the oxygen radical absorbance capacity assay (ORAC). Trolox, the aromatic part of vitamin E, was used as an internal standard and results are expressed as trolox equivalents (micromoles of trolox/micromoles of tested compound) on a comparative scale. Some commercially available indoles and indoleamines were included for comparison purposes to establish a subtractive structure-activity relationship between different moieties. Among all tested molecules, pinoline showed the highest antioxidant capacity

equivalent to 3-fold trolox activity, followed by **1** with a value identical to that melatonin. γ-Carboline **4** showed the lowest value among the non-acetylated carbolines. Unlike pinoline or **1**, its antioxidant potential is maintained when acetylated (**5**). The antioxidant potential of amides **2** and **3** diminished in up to one equivalent compared to parent compounds. Melatonin, equivalent to nearly 2.5 units of trolox, showed the highest ORAC value within the indoles and indoleamines included in the assay, followed by 5-methoxytryptamine (0.5 eq. difference) and 5-methoxyindole (1 eq. difference). Non-substituted indole showed significant antioxidant activity similar to that of trolox, whereas 1-methylindole was totally devoid of it at 10 μM (data not shown).

Table 3. Antioxidant Properties of Melatonin – Pinoline Hybrids **1**-**5** and Reference Compounds Determined in the Oxygen Radical Absorbance Capacity assay (ORAC, Trolox Equiv).[a]

Compd.	ORAC (trolox equiv)	Compd.	ORAC (trolox equiv)
1	2.39 ± 0.26	melatonin	2.43 ± 0.05
2	2.07 ± 0.19	5-MeOT	1.95 ± 0.04
3	1.38 ± 0.08	5-methoxyindole	1.60 ± 0.03
4	1.83 ± 0.08	indole	1.20 ± 0.02
5	1.67 ± 0.12	pinoline	3.07 ± 0.25

[a]Results are the mean ± SEM of 3 independent experiments.

Being **2** the sole compound able to stimulate a significant response in melatonin receptors, its neurogenic potential was determined in an *in vitro* model of neural stem

cells isolated from the adult rat subventricular zone (SVZ),[38] using melatonin as reference. TuJ-1 and MAP-2 expression was quantified and statistically analysed (one-way ANOVA analysis with drug treatment as the between-subject factor, followed by t-Student post hoc test). Our results show that both melatonin and **2** were able to promote neurogenesis in the model employed, whereas the melatonergic antagonist luzindole did not show any differences compared to control (Figure 2). **2**-Treated SVZ neurospheres (NS) express more TuJ1 (neuron-specific class III beta-tubulin) and MAP-2 (microtubule-associated protein 2) than those treated with melatonin. Melatonin-promoted TuJ1 expression occurred mainly in the outer part of the neurospheres, in cells spreading out of the formation, whereas the total expression of MAP-2 was diffuse within the neurosphere and occurred mostly in the soma of the neurons. TuJ1 expression pattern of **2**-treated SVZ-NS seemed to correspond to that of melatonin, more abundant in the outer parts and in cells spreading out of the NS, but the density of expression is comparatively higher. The expression of MAP-2 was dense and homogeneous both in the inner and the outer parts of the NS treated with **2**. Comparing the expression pattern of MAP-2 there were clear differences between melatonin and **2**. The expression of MAP-2 elicited by the latter was not limited to the neuronal soma but irradiated throughout the cell bodies in the proximal part of the neuronal fibbers.

The implication of melatonin receptors in the neurogenic properties of **2** was studied *in vitro.* When SVZ-NS were preincubated in the presence of the non-selective melatonin antagonist luzindole, the expression of both TuJ1 and MAP-2 was blocked. The blockade was near complete in the case of MAP-2, but some residual expression of TuJ1 remained despite the presence of the antagonist (Figure 2).

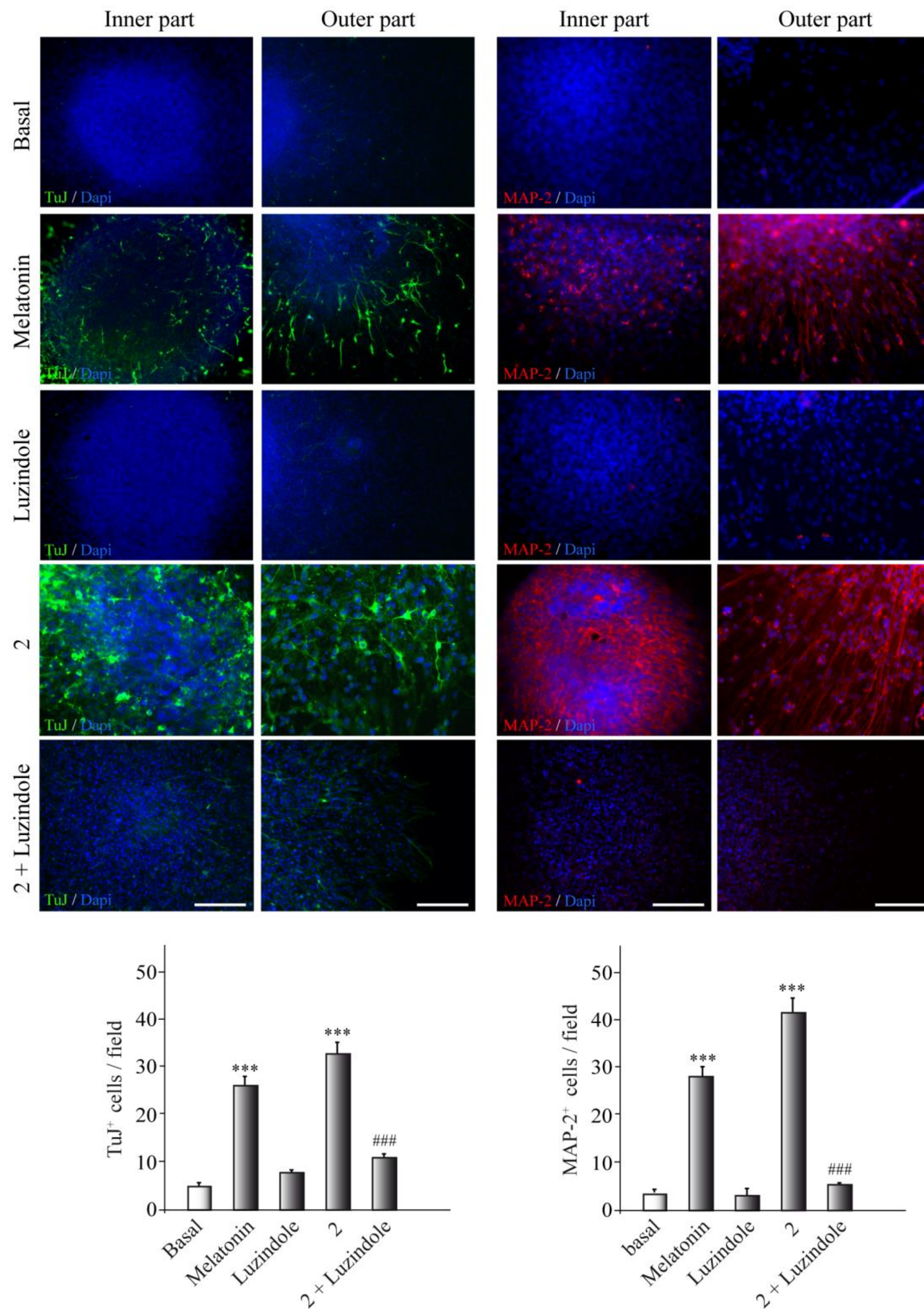


Figure 2. Immunostaining of neurogenic markers TuJ1 (green) and MAP-2 (red) in SVZ-NS in the presence of different compounds (10 µM) and effect of luzindole (10 µM) on the expression of neurogenic markers promoted by **2**. DAPI (blue) was used as nuclear marker. Scale bar, 200 µm. Quantification of TuJ^{+} and $MAP\text{-}2^{+}$ cells in neurospheres is shown. Values are the mean ± SD from five neurospheres per condition. $^{***}p \leq 0.001$ versus vehicle-treated (basal) cultures; $^{\#\#\#}p \leq 0.001$ versus the values obtained in compound **2**-treated cultures in absence of luzindole.

In a second assay, the neurogenic properties of pinoline were also evaluated *in vitro* in the same model. At 10 µM pinoline potently promoted the irradiated expression of both TuJ1 and MAP-2 showing a pattern similar to that of **2** (Figure 3). Pinoline was also evaluated at 10 nM as a preliminary study of its intrinsic neurogenic potential at trace concentrations. The expression of TuJ1 was little affected by the concentration of pinoline, whereas the density of MAP-2 clearly diminished at 10 nM being mostly located in the neuronal body.

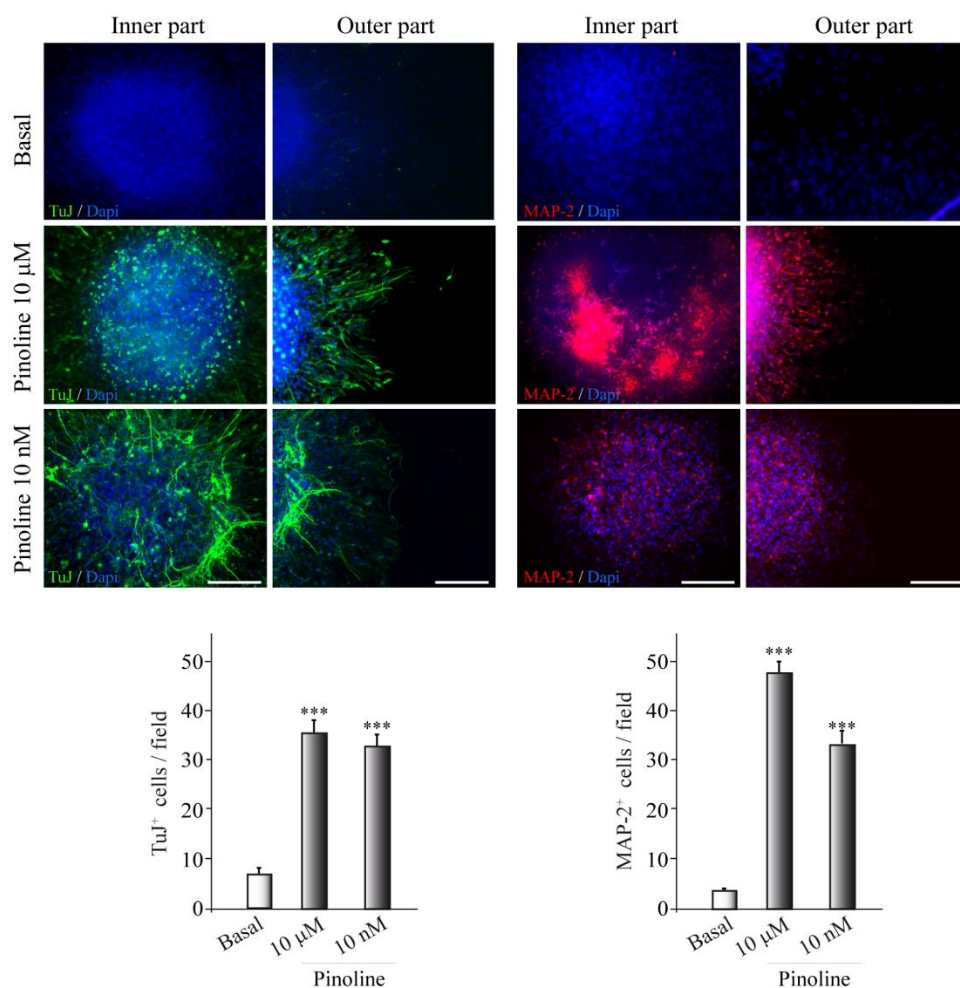


Figure 3. Immunostaining of neurogenic markers TuJ1 (green) and MAP-2 (red) in SVZ neurospheres in the presence of pinoline at different concentrations. DAPI (blue) was used as nuclear marker in all cases. Scale bar, 200 µm. Quantification of TuJ^+ and $MAP\text{-}2^+$ cells in neurospheres is shown. Values are the mean ± SD from five neurospheres per condition. $^{***}p \leq 0.001$.

DISCUSSION

Unlike pinoline and its isomers bearing an unsubstituted amino group (**1** and **4**), none of the acetylated compounds (**2**, **3**, and **5**) showed any activity at 5-HTRs due to the absence of a protonatable amine (Table 1). Binding affinities of pinoline and compound **1** were previously reported in rat 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors together with other β-carbolines.[39] Significant differences with their reported values were found only in the 5-HT_{2C}R. [^{3}H]Mesulergine was the radioligand employed in both studies, but in our case both pinoline and **1** showed more affinity for the human than for the rat 5-HT_{2C}R, being the difference around one order of magnitude.

Comparing pinoline and **1** we can conclude that the relative position of the methoxy group barely affects the affinity or the intrinsic activity of both β-carbolines at 5-HT_2R whereas the relative position of the basic nitrogen does; γ-carboline **4** is devoid of serotonergic activity in 5-HT_{2C}R (Table 2).

Herein we found that pinoline and **1** are both partial agonists at the 5-HT_{2A}R within the low micromolar range. Although direct 5-HTR stimulation by pinoline has long been speculated,[37, 40-42] to the best of our knowledge this is the first time that direct agonism is reported at the 5-HT_{2A}R for methoxylated-β-carboline structures. Glennon et al. studied some selected hallucinogenic β-carbolines, including harmala alkaloids, at the 5-HT_{2A}R and found no IP_3 (inositol-1,4,5-triphosphate) accumulation.[39] Agonism or partial agonism at 5-HT_{2A}R is needed to elicit psychedelic effects by classical hallucinogens; nevertheless not all agonists at this subtype possess such properties. Whether pinoline and/or **1** possess hallucinogenic properties at pharmacological concentrations remains unknown.[39] The described ability of pinoline to substitute for LSD in trained rats is scant, but this fact is a shared feature with reportedly

hallucinogenic harmala alkaloids, like harmine and harmaline.[43-45] Unlike other β-carbolines, such as the harmala alkaloid harmine (LD_{50} 38 mg/kg i.v. in mice), pinoline toxicity is rather low (LD_{50} 112 mg/kg i.v. in mice), although its administration to humans has never been reported in the scientific literature.[46, 47] Most harmala alkaloids potently inhibit the MAO-A isoform in the low nanomolar range,[22] whereas pinoline barely showed any significant monoamine oxidase inhibitory effect (MAO-A; IC_{50} = 41.5 ± 6.3 μM).[48] The MAO-A inhibition of pinoline could be of relevance at pharmacological doses but not if pinoline occurs in trace amounts under physiological conditions. The inhibitory effect in MAO-A is more pronounced in **1**, the nor-isomer of tetrahydroharmine. Both **1** (IC_{50} = 1.3 ± 0.3 μM) and tetrahydroharmine show a similar IC_{50} in the low micromolar range.[49]

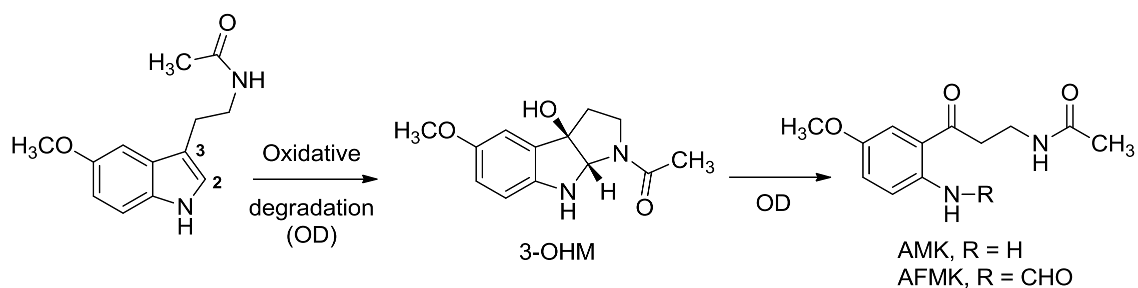
To what extent these results could be of relevance in putative endogenous-occurring pinoline remains unknown, in the same way the presence of endogenous pinoline is still controversial. Exogenous origin cannot be excluded, since several β-carbolines have been reported to be present in foodstuff.[50, 51] Regardless of the origin of the pinoline identified on the pineal gland by Langer et al., such low levels could still be of certain relevance in the stimulation of 5-HT_{2C}R. At this particular subtype, pinoline half maximal effective concentration (EC_{50} = 33 nM) is low enough to be of relevance upon its occurrence at trace concentrations.[8, 52] Previous studies revealed the presence of radiolabelled pinoline in neuronal membranes where 5-HTRs and SERT are located.[36] To the best of our knowledge, the careful determinations of pinoline concentration carried out by Langer et al. and more recently by Barker et al. in pineal gland have never been performed in adrenal gland; previous reports revealed a much higher concentration of pinoline in this gland than in the pineal body.[53, 54] This fact allows us to speculate that, even if the concentration observed could be above its real levels due to

the formation of artifacts, the presence of pinoline in the adrenal gland or that of related β-carboline structures with similar pharmacologic properties cannot be excluded.[13, 55] The effect of exogenous administration of pinoline in the hypothalamic-pituitary-adrenal axis has been described previously, generally associated to the stimulation of 5-HTRs.[46, 56] Intraperitoneal administration of pinoline to rats provokes effects that resembles those of tricyclic antidepressants, together with some anxiogenic-like effects.[57] Some β-carboline compounds related to harmala alkaloids are antagonists or inverse agonists at the benzodiazepine (BZD) binding site, producing anxiogenic effects. According to previous reports, pinoline lacks any affinity for the BZD binding site or the convulsive potential associated to its antagonism.[58, 59] However, anxiogenic effects are also observed after stimulation of 5-HT_{2C}R, which suggests that pinoline anxiogenic-like effects could be mediated by this receptor subtype.[60, 61] Our results in the serotonergic system call for a revision of the behavioural effects resulting from exogenous administration of pinoline; effects generally attributed to its inhibitory action of SERT and MAO-A.[62, 63] The behavioural effects caused by pinoline are more likely to be the result of an additive, if not synergic, intervention at all three systems.

Regarding to the radical scavenging properties of the studied compounds, the comparison of the ORAC values between pinoline and its 7-methoxy isomer **1** reveal that the relative position of the methoxy group seems to play a key role in the antioxidant properties of tetrahydro-β-carbolines. The observed difference is probably due to the differential radical/charge stabilization contribution of the lone pairs of the methoxy group in the oxidation process. Comparing pinoline and 5-MeOT, the antioxidant potential of the latter is 1 trolox eq. lower. 5-MeOT is essentially an open-ring pinoline what suggests that the plausible ring opening of the tetrahydropyrido cycle in the presence of oxidant species confers to the tricyclic molecule added antioxidant

potential compared to the lineal amine. The inverse structural analogy can be made between melatonin and **2**, being melatonin antioxidant potential slightly higher. This difference could be possibly related to the nature of the nitrogen in both pairs: amine, in the case of 5-MeOT/pinoline; and amide, in the case of melatonin/derivative **2**. The acetylation resulted in a dramatic reduction of the antioxidant potential of tetrahydro-β-carbolines (pinoline/**2**, **1**/**3**) but not in the case of tetrahydro-γ-carbolines (**4**/**5**). The only amine-amide pair in which acetylation results in improved antioxidant potential is in the case of 5-MeOT/melatonin.

Melatonin's chemical structure is known to intrinsically possess receptor-independent antioxidant properties.[64, 65] Cyclic 3-hydroxymelatonin (3-OHM) and other acyclic derivatives, such as N_1-acetyl-5-methoxy-kynuramine (AMK) and N_1-acetyl-N_2-formyl-5-methoxykynuramine (AFMK), are metabolites formed as the result of oxidative degradation of melatonin that showed also good radical scavenging activities.[66, 67] Their structures suggest that melatonin's antioxidant hotspot is located between positions C2 and C3 within the 5-methoxyindole (Scheme 2). Even though it does not seem obvious looking at the structure of such compounds, our data suggest that the acetamido group of melatonin should play a role in the oxidative degradation, i.e. stabilizing the tricyclic structure of 3-OHM, or in later phases of the scavenging cascade. Removal of the ethyl-acetamido group in melatonin or the tetrahydropyrido ring in pinoline leaves the bare 5-methoxyindole, the common heteroaromatic core of both compounds. This small scaffold is able to retain a significant antioxidant potential. Comparing indole and 5-methoxyindole, nearly one third of the antioxidant effect of the latter is attributable to the 5-methoxy group substitution. The contribution of the proton in position 1 seems to be even greater, being the 1-methylindole totally devoid of any antioxidant activity at testing concentration (10 μM).



Scheme 2. Melatonin oxidative degradation (OD) hotspots. Structure of cyclic 3-hydroxymelatonin (3-OHM) and kynuramines (AMK, AFMK).

Out of this antioxidant SAR no structural conclusions can be drawn to determine which decorations are best to enhance the reactivity in the presence of oxidant species. The antioxidant nature of pinoline and melatonin cannot be simply attributed to the reactivity of the 5-methoxyindole nucleus, nor envisioned as the result of a combination of chemical fragments that contribute separately to the whole antioxidant potential; instead, it suggests a more complex and optimized interaction between the moieties integrated around the heteroaromatic indole scaffold. Additional considerations should be made taking into account the nature of the ORAC assay. This experiment is able to measure the reactivity of a certain molecule against reactive oxygen species (ROS), but it is important to mention that *in vivo* other radicals are present as well, such as reactive nitrogen species for which this model it is not valid. Moreover molecules like melatonin, and probably pinoline, can mediate antioxidant responses within the cell via membrane receptors, interaction with transcription factors or by other mechanisms not directly related to the chemical reactivity of the molecule.[68] Pinoline has been proposed to have a direct role as DNA protecting agent against oxidative damage, and indeed some β-carbolines with antitumoral properties have demonstrated their ability to bind to it.[58] As abovementioned, radiolabelled pinoline was found to bind significantly to

mitochondria, where an eventual malfunction in the respiratory chain can result in the production of oxidative species.

Derivatives **2**, **3** and **5** are conformationally-restricted melatonin analogues. The only compound that showed significant affinity and activity at the melatonin receptors is the structurally orthodox melatonin – pinoline hybrid **2**. Compared to melatonin's subnanomolar affinity for MT_1 and MT_2 receptors, affinity of hybrid **2** was penalized by three orders of magnitude in both subtypes [K_i (MT_1) = 685 ± 25 nM; K_i (MT_2) = 330 ± 10 nM)]. Unlike some previously reported melatonin restricted analogues, in our case the rigidity through a secondary amide renders impossible a hydrogen bond.[69, 70] This interaction of the amide group with the receptor appears to be relevant at least for the MT_1 subtype.[71] As β-carboline **2** is a partial agonist at both receptors, its ability to activate both MT_1 and MT_2 suggests that the union of **2** to these receptors should occur in an analogous way to that of melatonin. None of the other melatonin restricted analogues reported in this work showed any activity at melatonergic receptors.

Compound **2** also demonstrated its ability to stimulate early neurogenesis and neuronal maturation in adult rat SVZ-NS to an extent comparable or superior to melatonin. The blockade of the expression of neurogenic markers exerted by luzindole, together with its demonstrated agonistic properties at the melatonin receptors allow us to infer that the neurogenic effect of **2** is mediated by MT_1 and MT_2 receptors. Both melatonin and pinoline, the two compounds embedded within the structural hybrid **2** demonstrated their *in vitro* neurogenic properties. In the case of melatonin its neurogenic effect can be effectively blocked likewise by luzindole.[30] Pinoline does not interact with MT_1 or MT_2 receptor, but is able to evoke agonistic response in serotonin receptors. The stimulation of serotonin receptors 5-HT_{1A} and 5-HT_2 is known to increase the neurogenesis rate in adult rat brain.[72, 73] Therefore, taking into account the

serotonergic pharmacological profile of pinoline we hypothesize that its neurogenic effect could be at least partially mediated by serotonin receptors.

Conclusions

We have synthesized and studied the SAR of a series of pinoline isomers and pinoline – melatonin hybrids able to penetrate into the central nervous system. We have characterized their pharmacological profile, which was compared to their parent amino compounds in different enzymes and receptor systems. This systematic study has allowed us to identify **1** and pinoline as full agonists at the 5-HT_{2C}R and partial agonists at the 5-HT_{2A}R. To the best of our knowledge, this represents the first report of β-carbolines resembling harmala alkaloids by exerting direct agonism at the 5-HT_{2A}R. Pinoline has been long known to be both a SERT and MAO-A inhibitor; our results demonstrate that it indeed binds to the serotonin transporter, and that it weakly inhibits MAO-A. Thus, direct action of pinoline on serotonergic receptors could be as relevant in its behavioural effects as the earlier known indirect effects via increased serotonin concentration, as the result of lowered serotonin metabolism or inhibited reuptake.

We have further studied the potential of melatonin and pinoline to act as direct radical scavengers in the presence of oxidant species. The results obtained reveal that both pinoline and melatonin are optimal antioxidant structures that cannot be envisioned as the sum of different moieties added over the 5-methoxyindole nucleus.

We have synthesized and characterized **2** as a structural and pharmacological hybrid between melatonin and pinoline that, despite lacking most of the pharmacological properties of pinoline, was able to partially retain some activity at melatonin receptors MT_1 and MT_2 and to a lesser extent, their direct scavenging

properties *in vitro*. Nevertheless the most prominent feature of this melatonin restricted analogue is its neurogenic potential *in vitro*; a response effectively blocked by luzindole and superior to that of melatonin. Taking into account its predicted ability to permeate through the blood-brain barrier, further studies *in vivo* would be required to determine if its potent neurogenic properties are also observed in the original cell niche.

Additionally we have demonstrated that pinoline was able to stimulate neurogenesis *in vitro* at low nanomolar concentration; an effect that could be, at least partially, mediated by its agonistic properties at serotonergic receptors. Be as it may, trace endogenous neurochemical or dietary β-carboline, pinoline neurogenic properties underscore its ability to potently interact with biological systems exhibiting an interesting pharmacological profile as an investigational tool, even at very low concentrations.

METHODS

Materials. Melatonin–pinoline hybrids (**1-5**) were obtained using described synthetic sequences, in good overall yields. All molecules gave satisfactory analytical (HPLC-MS) and spectroscopic data (^{1}H- and ^{13}C-NMR), in good agreement with their structures.

Assays for 5-HT Receptors and 5-HT Transporter. K_i determinations, agonist and antagonist functional data were generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, University of North Carolina at Chapel Hill, USA. Radioligands and cell lines employed for determining binding affinities were: [^{3}H]8-OH-DPAT in CHO (Chinese hamster ovary) cells stably expressing human 5-HT_{1A}R, [^{3}H]ketanserin in HEKT cells transiently expressing human 5-HT_{2A}R, [^{3}H]LSD in HEKT cells stably expressing human 5-HT_{2B}R, [^{3}H]mesulergine in Flp-IN HEKT cells stably expressing human 5-HT_{2C}R, [^{3}H]LSD in HEKT cells stably expressing human 5-HT_{7}R and [^{3}H]citalopram in HEKT cells stably expressing human SERT. Functional characterization of compounds in 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} was performed in transfected Flp-In HEK cells stably expressing the corresponding receptor subtype in the calcium mobilization FLIPR^{TETRA} assay. Detailed experimental protocols are described in the PDSP web site.[74]

Determination of human MAO Isoforms Activity. The potential effects of compounds on human MAO activity were investigated by measuring their effects on the production of hydrogen peroxide from *p*-tyramine, using the Amplex® Red MAO assay kit (Molecular Probes, Inc., Eugene, Oregon, USA) and microsomal MAO isoforms prepared from insect cells (BTI-TN-5B1-4) infected with recombinant baculovirus containing cDNA inserts for human MAO-A or MAO-B (Sigma-Aldrich). The

production of H_2O_2 catalysed by MAO isoforms was detected using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent) that reacts with H_2O_2 in the presence of horseradish peroxidase to produce the fluorescent product resorufin. In this study hMAO activity was evaluated following the general procedure previously described.[75]

Assays for MT_1 and MT_2 Receptor Subtypes. Binding and functional characterization of compounds in melatonergic receptors were carried out in transfected CHO cells stably expressing the human MT_1R or MT_2R, using 2-[^{125}I]iodomelatonin as radioligand and following described protocols.[76]

***In vitro* Blood–Brain Barrier Permeation Assay (PAMPA)**. Prediction of the brain penetration was evaluated using a parallel artificial membrane permeation assay (PAMPA-BBB) and porcine brain lipid (Avanti Polar Lipids) in a similar manner as previously described.[25, 77, 78]

Oxygen Radical Absorbance Capacity Assay. The ORAC-fluoresceine method was followed,[79] using a Polarstar Galaxy plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) with 485-P excitation and 520-P emission filters.

***In Vitro* Neurogenesis Studies**. Adult (8-12 weeks old) male Wistar rats (n = 6 per group), housed in a 12 h light-dark cycle animal facility, were used in this study. All procedures with animals were specifically approved by the Ethics Committee for Animal Experimentation of the CSIC and carried out in accordance with National (normative 1201/2005) and International recommendations (Directive 2010/63 from the European Communities Council). Special care was taken to minimize animal suffering. NS were derived from the SVZ of adult Wistar rats, which were induced to proliferate using established passaging methods to achieve optimal cellular expansion according to published protocols.[80, 81] After treatment, cells were processed for

immunocytochemistry with two types of neurogenesis-associated neuronal markers: TuJ1, associated with early stages of neurogenesis, and MAP-2, a marker of neuronal maturation. DAPI staining was used as a nuclear marker. Basal values were obtained under the same conditions, but in the absence of any product. The images were obtained using a Nikon fluorescence microscope 90i that was coupled to a digital camera Qi. The microscope configuration was adjusted to produce the optimum signal-to-noise ratio. The number of TuJ^+ and/or $MAP\text{-}2^+$ cells in the neurosphere was estimated from a total of five neurospheres per condition over three independent experiments as previously described.[82]

Statistical Determinations. One-way ANOVA analysis for comparisons between different treatments on neurospheres was performed using the SPSS statistical software package (version 20.0) for Windows (Chicago, IL) followed by Student's *t* post hoc test. Differences were considered statistically significant at $p < 0.05$.

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M.F.R. has contributed to the concept and design of the work, synthesis of compounds, PAMPA-BBB assays, analysis and interpretation of results, drafting and critical revision of the manuscript. C.P. has performed ORAC experiments and contributed to manuscript revision. J.A.M.-G. and S.A.-G. have performed the neurogenesis experiments and contributed to critical revision of the manuscript. A.P.-C. has supervised the neurogenesis studies and contributed to critical revision and approval of the manuscript. D.-H.C. has provided the experiments in melatonin receptors and contributed to critical revision and approval of the manuscript. M.Y. has determined the inhibition of human MAO enzymes and contributed to critical revision and approval of the manuscript. A.M.G. has performed some experiments on serotonergic receptors and contributed to critical review of the manuscript. M.I.R.-F. has contributed to the supervision of the work, drafting and critical revision of the manuscript, and approval of the manuscript.

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Supporting Information Available

Full experimental details for the synthesis of hybrids **1-5** and pharmacological protocols. This material is available free of charge via internet at http://pubs.acs.org.

Note

The authors declare no competing financial interest.

ABBREVIATIONS

AMK, N_1-acetyl-5-methoxy-kynuramine; AFMK, N_1-acetyl-N_2-formyl-5-methoxykynuramine; BZD, benzodiazepine; DAPI, 4',6-diamidino-2-phenylindole; 5-HT, 5-hydroxytryptamine or serotonin; 5-HTRs, serotonergic receptors; MAO, monoamine oxidase; MAP-2, microtubule-associated protein-2; 5-MeOT, 5-methoxytryptamine; MTRs, melatonergic receptors; NS, neurospheres; OD, oxidative

degradation; 3-OHM, cyclic 3-hydroxymelatonin; ORAC-FL, oxygen-radical absorbance capacity by fluorescence; PAMPA-BBB, parallel artificial membrane permeation assay for the blood-brain barrier permeation; ROS, reactive oxygen species; SERT, serotonin transporter; SVZ, subventricular zone; TuJ1, anti-β-III-tubulin antibody.

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