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Short Communication

Evaluating the effects of 2-BFI and tracizoline, two potent I_2-imidazoline receptor agonists, on cognitive performance and affect in middle-age rats

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Abstract

Purpose Cognitive performance and affective-like responses are particularly susceptible to decline during the aging process. Since imidazoline receptors I_2 are altered in neurodegenerative and psychiatric disorders, the pharmacological modulation of I_2 receptors could be of potential interest to improve these age-related behavioral deficits. In this context, the present study explored the potential pro-cognitive as well as antidepressant-like effects of two potent I_2 agonists, 2-BFI (2-(2-benzofuranyl)-2-imidazoline), and tracizoline (2-styryl-4,5-dihydro-lH-imidazole; also known as LSL 61122 or valldemossine) in middle-age male rats. *Methods* Following a preliminary study performed to select a dose regimen capable of inducing neuroplastic events (i.e., regulation of hippocampal FADD cell fate adaptor), 2-BFI or tracizoline (10 mg/kg, i.p.) was administered to 9-10 months old Sprague-Dawley rats. Cognitive performance was evaluated in the 8-arms radial maze and antidepressant-like responses under the stress of the forced-swim test. *Results* At the dose selected, 2-BFI and tracizoline did not alter body weight or cognitive performance. Interestingly, 2-BFI (but not tracizoline) induced hypothermia over the course of treatment and an antidepressant-like response in the forced-swim test. *Conclusion* These compounds did not improve cognitive performance in middle-age rats; however, 2-BFI exerted an antidepressant-like response. These results are in line with prior reports suggesting a beneficial impact induced by certain I_2 ligands on affective-like behavior, while adding that the beneficial effects were mediated by increasing climbing behavior and with the novelty and importance of demonstrating this effect for middle-age rats.

Keywords

Aging; cognition; depression; I_2 agonists; behavior; rat

Introduction

Since life expectancy is increasing, the elderly population is growing, and consequently, longevity comes at a price that includes a higher rate of health and functional problems. For example, during the aging process, both for humans and laboratory rodents, some key behavioral features are particularly vulnerable to decline, such as cognitive performance (Mattson and Magnus, 2006; Rosenzweig and Barnes, 2003) and affect (Herrera-Pérez et al., 2008; Mckinney and Sibille, 2013). A prior study from our research group evaluated how cognitive performance and affective-like behavior were impacted with age in male Sprague-Dawley rats, in order to set up a time frame in which to explore new therapeutic candidates to improve these behavioral age-related outcomes (i.e., with some cognitive and affective declines observed starting at middle-age and onward) (Hernández-Hernández et al., 2018a). In search for new potential pharmacological targets, a recent review by Bousquet et al. (Bousquet et al., 2020) extensively covered the past, present and future of the imidazoline receptor (IR) system. This system, which is comprised by three known IRs (I_1, I_2 and I_3), is situated in various locations including the brain (for I_1 and I_2), and is involved in many key functions (see Bousquet et al., 2020 and references within). From all IRs, I_2 receptors emerge as the most promising neuropharmacological targets (see Bousquet et al., 2020), since their dysregulation (change in receptor density and/or in its response) have been associated, among other brain disorders, with neurodegenerative (e.g., increased receptor I_2 density in cortical membranes of *postmortem* brains from patients with Alzheimer's disease, see García-Sevilla et al., 1999) and psychiatric disorders (e.g., decreased receptor I_2 density in suicide victims of major depressive disorders, García-Sevilla et al., 1996a,b). Thus, the pharmacological modulation of I_2 receptors present as a promising and underestimated/neglected target to improve the cognitive decline and depressive-like phenotype associated with aging, since they have proven beneficial effects in young rats for both behavioral responses.

In fact, some I_2 selective agonists, besides inducing hypothermia and neuroprotection (see revision in Bousquet et al., 2020), were capable of ameliorating cognitive impairment in rodent models (see Griñán-Ferré et al., 2019; Abás et al., 2020, for studies done in collaborative experiments from our research group). Moreover, 2-BFI (2-(2-benzofuranyl)-2-imidazoline), the most studied I_2 agonist, significantly improved learning and spatial memory and navigation abilities in a rat model of Alzheimer's disease as evaluated in the water maze, and it did so in a dose-dependent manner (Tian et al., 2017). As for tracizoline, to the best of our knowledge, no prior studies have evaluated its effects over cognitive performance at any age in rodents. Conversely, when revising the literature regarding the potential affective-like responses of I_2 agonists, the number of prior studies is not only limited for young rats but also contradictory. Whereas various studies described antidepressant-like effects (assessed under the stress of the forced-swim test) mediated by certain I_2 agonists (2-BFI in rats: Nutt et al., 1995, and mice: Tonello et al., 2012; BU224 in rats: Finn et al., 2003; CR4056 in rats: Siemian et al., 2019), others showed no beneficial effects (BU224, BU239, BDF 8082 in mice: O'Neill et al., 2001; 2-BFI, phenyzoline in rats: Siemian et al., 2019; LSL 60101 in rats: Hernández-Hernández et al., 2020). Again, there is no prior record evaluating the antidepressant-like potential of tracizoline in rats with aging.

Against this background, the present study evaluated the potential pro-cognitive as well as antidepressant-like responses of 2-BFI in middle-age male rats, while it explored, for the first time, the putative behavioral responses induced by tracizoline (2-styryl-4,5-dihydro-lH-imidazole; also known as LSL 61122 or valldemossine; see Bousquet et al., 2020 and references within for prior ascribed effects to this compound).

Material and methods

Animals

A preliminary experiment was performed in male Sprague-Dawley rats (6 or 12 months old, n = 30, 15 per age group; see Fig. 1A) to select the dose of 2-BFI or tracizoline to be later administered to evaluate the behavioral responses. To do so, a total of 22 middle-age (9-10 months old; see Hernández-Hernández et al., 2018a, for emerging behavioral deficits at this age-window) male Sprague-Dawley rats were used (Fig. 2A). All rats were bred in the animal facility at the University of the Balearic Island. In an attempt to reduce the number of rats used, since prior experiments from our research group extensively compared young vs. middle-age rats (i.e., ages from 3-13 months old) in terms of their behavioral cognitive and affective decline observed with age, in the present study we selected one age of study (9-10 months old). Moreover, our previous study showed decreased levels of the brain cell adaptor FADD with age starting at 6 months old in male rats (Hernández-Hernández et al., 2018a). Following animal housing regulations regarding the number of animals allowed per cage in terms of size and weight, rats of this age had to be singled housed in standard cages under specific environmental conditions (22 °C, 70% humidity, 12-h light/dark cycle, lights on at 8:00 AM) with *ad libitum* access to a standard diet and tap water (except when otherwise specified, see Fig. 2A for food deprivation regimens prior to radial maze testing). All animal experiments were performed during the light period (between 8:00 AM and 15:00 PM) and adhered to ethical standards. The care of animals was performed following the ARRIVE guidelines (McGrath and Lilley, 2015) in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU, under which the study was performed, and were approved by the Local Bioethical Committee (University of the Balearic Islands) and the regional Government (Conselleria Medi Ambient, Agricultura i Pesca, Direcció General Agricultura i Ramaderia, Govern de les Illes Balears). All efforts were made to minimize the number of rats used and their suffering.

Establishing a putative neuroprotective dose regimen for 2-BFI and tracizoline in middle-age rats

Rats from two ages (6 and 12 months old) were handled prior to being allocated in 3 treatment groups that received either 1 day or 7 days 2-BFI HCl (1x or 7x 10 mg/kg, i.p., n = 5 per age and dose regimen), tracizoline HCl (1x or 7x 10 mg/kg, i.p., n = 5 per age and dose regimen) or saline (7x 1 ml/kg of 0.9% NaCl, i.p., n = 5 per age; see Fig. 1A for more details). 2-BFI and tracizoline were kindly provided by Ipsen Pharma, Barcelona, Spain. Treatment doses were selected from prior studies in young rats (2-3 months) from our group that tested several doses (e.g., Garau et al., 2013) to show that 2-BFI and/or tracizoline induce certain neuroprotective actions at this dose (e.g., Garau et al., 2013; Keller and García-Sevilla, 2016). Rats were killed on D8 and the hippocampus was freshly dissected and frozen to evaluate for putative neuroprotective actions induced by the cell fate adaptor FADD by western blot analysis as previously described in detail by our group (e.g., Hernández-Hernández et al., 2018a; Ramos-Miguel et al., 2017; Hernández-Hernández et al., 2018b), these references and the references within describe in detail the putative role of FADD adaptor in terms of its neuroplasticity *vs* apoptotic actions; see also Ramos-Miguel et al., 2012 for FADD relevant features and its immunodetection characterization with this particular antibody). In the present experiment, total proteins (40 μg) from hippocampal homogenates were run in SDS-PAGE minigels (Bio-Rad Laboratories, CA, USA), transferred to nitrocellulose membranes and incubated overnight with a blocking solution containing anti-FADD (H-181) antibody (dilution 1:5000, sc-5559, Santa Cruz Biotechnology, CA, USA). The next day, membranes were incubated with anti-rabbit immunoglobulin G linked horseradish peroxidase and FADD signal was detected with ECL reagents (Amersham, Buckinghamshire, UK), visualized with an autoradiographic film (Amersham ECL Hyperfilm) and quantified by densitometric scanning (GS-800 Imaging Calibrated Densitometer, Bio-Rad). The amount of target protein

for each treatment group was detected in the same gel with that of its respective age-control group. The quantification procedure was repeated at least 3 times in different gels and the mean value was used as a final estimate. All experiments were reblotted and reprobed for β-actin (dilution 1:10000, clone AC-15, Sigma Chemical) which was quantified as a loading control.

Exploring the potential effects of 2-BFI and tracizoline on body temperature, cognitive performance and affect in middle-age rats

As described in the experimental design in Fig. 2A, all rats were handled and weighted for 2 days prior to any manipulations. Rats were randomly allocated in 3 groups that were treated daily for 7 days with either 2-BFI HCl (7x 10 mg/kg, i.p., n = 7), tracizoline HCl (7x 10 mg/kg, i.p., n = 7), or saline (7x 1 ml/kg of 0.9% NaCl, i.p., n = 8). Body weight and core body temperature, which was recorded basally and 1 h post-treatment by a rectal probe connected to a digital thermometer (Compact LCD display thermometer, SA880-1M, RS, Corby, UK), were monitored daily throughout treatment.

Cognitive performance was evaluated in the 8-arms radial maze 1 h after the first treatment dose (D1, 1x 10 mg/kg) and 24 h after the last treatment dose (D8, repeated effects following 7x 10 mg/kg) as previously described (Hernández-Hernández et al., 2018b; in which pro-cognitive effects were observed with no pretraining conditions). Since this behavioral test requires motivation for food, rats were food-deprived for 48 h before performing each test (2 days before acute treatment and during D6 and D7 of treatment, see Fig. 2A). The 8-arms radial maze (Panlab, SL, Barcelona, Spain), located in a room with several external visual cues, consists of 8 equally spaced radial arms (50 cm long x 12 cm wide) with a food reward (pellet) at the end of each arm. Each test day, rats were allowed to freely visit the arms (one session per test day) to obtain food pellets until they completed the maze (visited all 8-arms)

or the duration of the test ended (20 min). The behavioral equipment was cleaned in between animals. Individual test sessions were videotaped and animal movement was monitored via a digital video tracking system (LE 8300 with software SEDACOM v 1.3, Panlab, SL, Barcelona, Spain). The amount of time needed (sec) and the number of errors committed (sum of reentered and non-visited arms) to complete the maze was examined blindly to treatment group assignment and results were used as an indicative of each rat's spatial working memory performance.

Following 4 days of rest, the potential antidepressant-like responses exerted by 2-BFI or tracizoline were evaluated following standard procedures (e.g., Cryan et al., 2002) in the forced-swim test as previously described for our group (see García-Fuster and García-Sevilla, 2016). Briefly, on pre-test day (D12, see Fig. 2A) rats were forced to swim for 15 min in individual tanks (41 cm high x 32 cm diameter) filled with water (25 cm of depth, 25 ± 1 ºC). After the pre-test phase, rats were treated with 3 doses over the course of 24 h of 2-BFI (3x 10 mg/kg, i.p.), tracizoline (3x 10 mg/kg, i.p.) or saline (3x 1 ml/kg, i.p.), 22, 4 and 1 h prior to the actual 5 min test (D13), which was videotaped. Clean water tanks were used for each rat. Videos were blindly analyzed (Behavioral Tracker software, CA, USA) to obtain the time (sec) spent immobile or active (i.e., climbing or swimming).

Data and statistical analysis

The software GraphPad Prism, Version 8 (GraphPad Software, CA, USA) was used for data and statistical analysis. Results, expressed in circles or bar graphs, represent mean values ± standard error of the mean (SEM). Individual symbols for each rat are shown within bar graphs. Changes in body weight, body temperature and cognitive performance (8-arms radial maze) were assessed by two-way repeated measures (RM) ANOVAs followed by Sidak's multiple comparison tests when appropriate, in which Treatment and Day were used as

independent variables. Data from the forced-swim tests were analyzed by one-way ANOVAs followed by Sidak's when applicable. Normal distribution for each measurement reported was evaluated with Shapiro-Wilk normality test. The level of significance was set at $p \leq 0.05$.

Results

Putative neuroprotective dose regimen for 2-BFI and tracizoline in middle-age rats

The present results showed no age-differences (6 vs. 12 months of age) in terms of hippocampal FADD regulation, suggesting as previously reported decreased FADD content at these ages, in which to evaluate the effects of each I_2 pharmacological treatment. Data was analyzed combining both age groups (6 + 12 months of age: n = 10 per treatment group, see Fig. 1B-C; as shown in Supplemental data for Fig. 1). The overall results showed increased FADD content following 7x 10 mg/kg (but not 1x 10 mg/kg) treatment with 2-BFI (+27 ± 10%, $*p < 0.05$; Fig. 1B) and tracizoline (+19 ± 11%, $*p = 0.05$; Fig. 1C) when compared to control: saline-treated rats.

2-BFI or tracizoline did not alter body weight over the course of treatment

When analyzing the effects of 2-BFI and tracizoline on body weight (see Fig. 2B; see original source data on Supplemental data for Fig. 2), while a two-way RM ANOVA detected a significant effect of Day ($F_{6,114} = 21.95$, $p < 0.001$), there was no effect of Treatment ($F_{2,19} = 0.13$, $p = 0.882$), nor a Treatment x Day interaction ($F_{12,114} = 1.23$, $p = 0.273$). Notice that the fluctuations in body weight observed for all groups are due to food deprivation (48 h) prior to radial maze testing (see Fig. 2B).

2-BFI, but not tracizoline, induced hypothermia over the course of treatment

When analyzing the effects of 2-BFI and tracizoline on core body temperature (see Fig. 2C; see original source data on Supplemental data for Fig. 2), a two-way RM ANOVA detected a significant effect of Day ($F_{6,114} = 26.79$, $p < 0.001$), Treatment ($F_{2,19} = 13.50$, $p < 0.001$), and a significant Treatment x Day interaction ($F_{12,114} = 2.95$, $p < 0.01$). *Post-hoc* analysis revealed that 2-BFI, but not tracizoline, induced hypothermia from days 2 to 5 of treatment (drop in temperature ranging from -0.8 to -1.4 °C; *$p < 0.05$ and ***$p < 0.001$ vs. control: saline-treated rats; Fig. 2C), and induced tolerance to the hypothermic effect following 6 days of treatment (see Fig. 2C).

2-BFI or tracizoline did not change cognitive performance over the course of treatment

The possible effects of 2-BFI and tracizoline treatments on cognitive performance were evaluated by the time needed (sec) to complete the maze and the number of errors performed (see Fig. 2D; see original source data on Supplemental data for Fig. 2D). The results, as evaluated by two-way RM ANOVAs, showed no significant effects of Day (time: $F_{1,19} = 0.03$, $p = 0.861$; number of errors: $F_{1,19} = 0.80$, $p = 0.383$), Treatment (time: $F_{2,19} = 0.69$, $p = 0.514$; number of errors: $F_{2,19} = 0.30$, $p = 0.743$), Treatment x Day interaction (time: $F_{2,19} = 2.60$, $p = 0.101$; number of errors: $F_{2,19} = 0.01$, $p = 0.987$).

2-BFI, but not tracizoline, induced an antidepressant-like response in middle-age rats

When evaluating the effects of 2-BFI and tracizoline treatments (3x 10 mg/kg over a 24-h period) on the time spent immobile (sec) in the FST, a one-way ANOVA detected significant differences ($F_{2,18} = 3.61$, $p < 0.05$; Fig. 2E). *Post-hoc* analysis revealed that 2-BFI, but not tracizoline, significantly reduced immobility (-41 ± 15 sec; *$p < 0.05$ vs. control: saline-treated rats; Fig. 2E; see original source data on Supplemental data for Fig. 2E). Interestingly, the decrease in immobility could be explained by an increase in the time spent climbing

($F_{2,18}$= 3.25, p = 0.06), since *post-hoc* comparisons revealed that 2-BFI increased climbing (+44 ± 17 sec; *p < 0.05 vs. control: saline-treated rats; Fig. 2E). No overall changes were observed for swimming behavior (data not shown).

Discussion

The present study evaluated whether 2-BFI or tracizoline, two potent I_2 agonists, could improve cognitive performance and affective-like behavior in middle-age rats at a dose that induced certain degree of neuroprotection in the aged hippocampus. While neither drug treatment altered body weight, 2-BFI induced hypothermia over the course of treatment. Moreover, although the results showed no effects over cognitive performance, 2-BFI (but not tracizoline) induced an antidepressant-like effect under the stress of the forced-swim test in middle-age rats.

Selective I_2 ligands with ascribed agonist properties dose-dependently induce acute hypothermia in rats (effects prevented by idazoxan, an I_2 receptor antagonist), including 2-BFI and tracizoline (Thorn et al., 2012), and other structurally novel I_2 ligands in mice (see Abás et al., 2017; Griñán-Ferré et al., 2019; Abás et al., 2020). Moreover, the induction of tolerance to the hypothermic effects of I_2 drugs following repeated drug exposure has been previously shown (Abás et al., 2017; Griñán-Ferré et al., 2019; Abás et al., 2020). In line with this, 2-BFI (daily dose of 10 mg/kg) initially reduced core body temperature (observed for up to 5 days) to later result in the induction of tolerance, as previously suggested for other I_2 ligands in younger rats. In this line of thought, it has been proposed that some of the neuroprotective effects of I_2 selective agonists may be related to hypothermic effects of the drug. Contrarily, the present study failed to show the induction of hypothermia by tracizoline (daily dose of 10 mg/kg) in middle-age male rats, thus suggesting that a higher dose of

tracizoline might be needed to induce hypothermia as previously described (e.g., doses of 32 or 56 mg/kg; Thorn et al., 2012).

A prior report from our research group showed that hippocampal FADD decreased with age in rats, with a peak decrease observed starting at 6 months of age which kept constant later on (i.e., 13 moths of age; Hernández-Hernández et al., 2018a). Moreover, multifunctional FADD protein decreased in the brain with age in parallel to deficits in cognitive performance in rats (Hernández-Hernández et al., 2018a) and in an elderly community sample (Ramos-Miguel et al., 2017). Therefore, since repeated treatments with UK-14304 (an α_2-adrenergic agonist) increased FADD while improved cognitive performance in aged rats (Hernández-Hernández et al., 2018b), the observed increase in hippocampal FADD by 2-BFI and tracizoline following 7x 10 mg/kg of treatment suggested that this dose paradigm could be a great starting point to evaluate the potential pro-cognitive and antidepressant-like effects of these drugs in middle-age rats. In this context, prior data suggested that particular I_2 selective agonists could improve cognitive performance in rodents (Abás et al., 2020; Griñán-Ferré et al., 2019), including a potential benefit for 2-BFI (Tian et al., 2017), the present results failed to observe such an improvement, as measured by the time needed (sec) and number of error committed to resolve the 8-arms radial maze at the doses tested (1x and 7x 10 mg/kg on D1 and D8 respectively). However, many discrepancies existed between the present and the prior study (Tian et al., 2017) that could explain the differences in 2-BFI responses. For example, Tian et al. (2017) utilized 2 months old rats (vs. 9-10 months old rats in the present study); injected with Aβ1-42 into hippocampal CA1 (Cornu Ammonis) region to model the cognitive decline observed in Alzheimer's disease (vs. naïve middle-age rats in the present study); and evaluated learning and memory abilities in the water maze (vs. 8-arms radial maze with no prior training). Moreover, these drugs may selectively improve some aspects of cognitive function, but not others (e.g., spatial memory),

and thus the 8-arms maze might not capture the specific efficacy. In favor of our results, the efficacy of the 8-arm radial maze (same protocol used here) demonstrating an age-related cognitive decline was previously validated by our group in middle-age rats, and was also corroborated with other behavioral test (e.g. Barnes maze, see Hernández-Hernández et al., 2018a,b). In fact, our prior data showed a positive cognitive improvement induced by a different pro-cognitive drug (i.e., UK-14304, an α-2 adrenoceptor agonist) when following the same administration protocol in middle-aged rats (see Hernández-Hernández et al., 2018b). Plus, given that 2-BFI was beneficial in a dose-dependent manner (Tian et al., 2017), maybe higher doses are needed for 2-BFI to improve cognitive performance in the 8-arms radial maze as compared to the doses needed to induce neuroprotection, since neuroplastic changes may not always correlate directly with behavior. Nevertheless, since no prior data was available for tracizoline effects over cognitive performance at any age in rodents, the present results are novel and suggest that, at least at the doses tested (D1: 10 mg/kg and D8: 7x 10 mg/kg) and with middle-age rats, tracizoline does not seem to induce pro-cognitive responses, and also the need for future studies with other doses and/or treatment paradigms to further ascertain tracizoline effects. Although no positive control was run in parallel to the drugs tested, a recent study from our group extensively characterized the decline in cognitive performance with age in relation to FADD regulation and showed that 8-OH-DPAT was capable of inducing a pro-cognitive effect in middle-age rats (Hernández-Hernández et al., 2018a), ensuring optimal conditions for our behavioral settings. Similarly, in our hands, UK-14304 improved cognitive performance in the 8-arms radial maze in middle-age rats (Hernández-Hernández et al., 2018b), therefore demonstrating that the lack of positive effects is not due to problems related to the behavioral set-up.

The present results demonstrated an antidepressant-like potential for 2-BFI in middle-age rats, in line with prior positive data for this I_2 ligand in younger rodents (Nutt et al., 1995;

Tonello et al., 2012). It is worth mentioning that the antidepressant-like effect was observed after 3 doses of 10 mg/kg of 2-BFI administered over a 24 h period between pre-test and test day in the forced-swim test (see García-Fuster and García-Sevilla, 2016), similarly to all prior published data. Interestingly, reduced immobility was driven by increased climbing behavior as expected for antidepressants that enhance norepinephrine neurotransmission (see Detke et al., 1995, 1997; Rénéric et al., 2002) and in line with prior reports suggesting I_2 ligands increase noradrenaline in the brain (e.g., Siemian et al., 2018; Barrot et al., 2000) and/or mediate some of their responses (e.g., Siemian et al., 2018). This improvement on climbing is novel since prior studies evaluating I_2 ligands in the forced-swim test only measured immobility (Nutt et al., 1995; Tonello et al., 2012; Siemian et al., 2019), except (Finn et al., 2003) that showed that decreased immobility was paired with an increase in mild swimming behavior. Although it was proposed that I_2 receptor activation stimulates locomotion activity in rats (Barrot et al., 2000), the potential locomotion-stimulating effects of 2-BFI were evaluated at the same dose tested here (10 mg/kg, and even at higher doses) in a recent study in Sprague-Dawley rats (Thorn et al., 2012), showing no changes in locomotion, and therefore ensuring that the effects observed in the forced-swim test might not be driven by hyperlocomotion. Moreover, the administration of tracizoline did not induce any changes in affective-like behavior as measured in the forced-swim test. These data complement the pool of negative data for other I_2 ligands (Siemian et al., 2019; O'Neill et al., 2001; Hernández-Hernández et al., 2020), and further propose that only specific I_2 compounds could induce particular behavioral effects (as previously implied and revised; see revision in Bousquet et al., 2020, and references within), thus suggesting the need for future studies evaluating the key distinctions among ligands (and/or their interaction with I_2 receptor) that drive these differential effects.

To conclude, the modulation of I_2 receptors with specific I_2 agonists, such as 2-BFI and tracizoline, did not result as expected in terms of improving the cognitive decline and depressive-like phenotype associated with aging in rats. However, while the present results showed that a dose paradigm capable of inducing neuroprotective actions did not improve cognitive performance, it demonstrated a promising antidepressant-like potential for 2-BFI in middle-age rats. Given the distinct behavioral effects induced by particular I_2 ligands and the particularities of each experimental design (species, dosage, tests performed), future studies should further explore the potential benefit of targeting I_2 receptors to improve the behavioral deficits associated with aging (including the progression from middle- to old-age). These studies should be design to overcome the limitations of the present study, such as, for example, including more doses, testing drugs under experimental-created cognitive deficits, and/or evaluating other aspects of cognitive function (multiple behavioral tests for cognitive performance).

Declarations

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Conflict of interest

None.

Data availability

Not applicable.

Authors' contributions

EH-H and MJG-F designed the study. EH-H performed the behavioral testing and analysis. MJG-F wrote the first draft of the manuscript. Both authors contributed to and approved the final version of the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

Ethical approval

All animal experiments adhered to ethical standards. The care of animals was performed following the ARRIVE guidelines (McGrath and Lilley, 2015) in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU, under which the study was performed, and were approved by the Local Bioethical Committee (University of the Balearic Islands) and the regional Government (Conselleria Medi Ambient, Agricultura i Pesca, Direcció General Agricultura i Ramaderia,

Govern de les Illes Balears). All efforts were made to minimize the number of rats used and their suffering.

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Legends for Figures

Fig. 1. Establishing a putative neuroprotective dose regimen for 2-BFI and tracizoline in aged rats. A Experimental design. Treatment Groups: Control (saline-treated rats: 0.9% NaCl, 7x 1 ml/kg, i.p., 1 injection per day, n = 5 per age), 2-BFI HCl (1x or 7x 10 mg/kg, i.p., 1 injection per day, n = 5 per age) and tracizoline HCl (1x or 7x 10 mg/kg, i.p., 1 injection per day, n = 5 per age). Abbreviations: D, day. **B** FADD protein content in hippocampal samples following 2-BFI and tracizoline treatments on D8. Columns represent mean ± SEM of n experiments per group and expressed as a percentage of their respective age-control (individual values are shown for each rat in symbols). *$p \leq 0.05$ when compared to control (saline-treated by Student's *t*-tests) rats (combined analysis for 6 and 12-months old rats). Representative immunoblots depicting the labeling of FADD and loading control β-actin for each age and treatment group are shown below. Full-lengths immunoblots with molecular mass markers are shown in Supplemental Fig. 1.

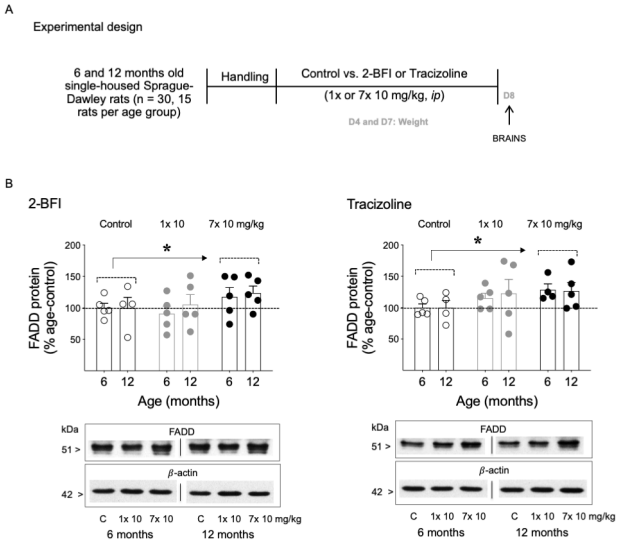


Fig. 2. Exploring the potential effects of 2-BFI and tracizoline on body temperature, cognitive performance and antidepressant-like response in adult male aged rats. A Experimental design. Treatment Groups: Control (saline-treated rats: 0.9% NaCl, 1 ml/kg at the indicated times, i.p., n = 8), 2-BFI HCl (10 mg/kg at the indicated times, i.p., n = 7) and tracizoline HCl (10 mg/kg at the indicated times, i.p., n = 7). Abbreviations: D, day; FST, forced-swim test. **B** Body weight measurements across days. Circles represent mean ± SEM of body weight (g). A two-way RM ANOVA did not detect any changes. **C** Core body temperature across treatment days. Circles are mean ± SEM of the difference (Δ, 1 h, basal value) in body temperature (°C). A two-way RM ANOVA followed by Sidak's *post-hoc* comparisons: ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$ vs. control: saline-treated rats. **D** Time

needed (sec) and number of errors committed in the 8-arms radial maze on D1 and D8. Circles represent mean ± SEM of the particular behavior. Two-way RM ANOVAs did not detect any significant changes. **E** Time spent immobile (sec) or climbing (sec) in the FST on D13. Columns represent mean ± SEM of the time spent in each behavior (sec). Individual values are shown in symbols for each rat. One-way ANOVA followed by Sidak's *post-hoc* test: *$p < 0.05$ vs. control: saline-treated rats.

