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1-cyclohexyl-x-methoxybenzene derivatives, novel psychoactive substances seized on the internet market. Synthesis and in vivo pharmacological studies in mice

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Abstract

Introduction Among novel psychoactive substances notified to EMCDDA and Europol were 1-cyclohexyl-x-methoxybenzene stereoisomers (*ortho*, *meta*, and *para*). These substances share some structural characteristics with phencyclidine and tramadol. Nowadays, no information on the pharmacological and toxicological effects evoked by 1-cyclohexyl-x-methoxybenzene are reported. The aim of this study was to investigate the effect evoked by each one stereoisomer on visual stimulation, body temperature, acute thermal pain, and motor activity in mice.

Methods Mice were evaluated in behavioral tests carried out in a consecutive manner according to the following time scheme: observation of visual placing response, measures of core body temperature, determination of acute thermal pain, and stimulated motor activity.

Results All three stereoisomers dose-dependent inhibit visual placing response (rank order: *meta* > *ortho* > *para*), induce hyperthermia at lower and hypothermia at higher doses (*meta* > *ortho* > *para*) and cause analgesia to thermal stimuli (*para* > *meta* = *ortho*), while they do not alter motor activity.

Conclusions For the first time, this study demonstrates that systemic administration of 1-cyclohexyl-x-methoxybenzene compounds markedly inhibit visual response, promote analgesia, and induce core temperature alterations in mice. This data, although obtained in animal model, suggest their possible hazard for human health (i.e., hyperthermia and sensorimotor alterations). In particular, these novel psychoactive substances may have a negative impact in many daily activities, greatly increasing the risk factors for workplace accidents and traffic injuries.

KEYWORDS

1-cyclohexyl-x-methoxybenzene, behavior, body temperature, mice, Novel Psychoactive Substances, tail withdrawal

1 | INTRODUCTION

Over the past 5 years, there has been an unprecedented increase in the number, type, and availability of novel psychoactive substances (NPS) in Europe that are sold openly as "legal" replacements for illicit drugs. Continuing this trend, during 2015, a total of 100 new substances (i.e., cathinones, cannabinoids, phenethylamines, opioids, tryptamines, benzodiazepines, arylalkylamines, and other groups) were reported for the first time to the EU Early Warning System, bringing the total number of new substances monitored to more than 560, with more than 380 (70%) of these detected in the last 5 years alone and with two new substances detected every week (EMCDDA, 2016). With increased availability, harms have increased, such as acute, sometimes fatal, poisonings (Chiappini et al., 2015; Loi et al., 2015; Dines et al., 2015; Zawilska & Andrzejczak, 2015; Bersani et al., 2014;

*Equally contributed to the experimental work.

Abbreviations: ortho, 1-cyclohexyl-2-methoxybenzene; meta, 1-cyclohexyl-3-methoxybenzene; para, 1-cyclohexyl-4-methoxybenzene; NPS, Novel Psychoactive Substances; PCP, Phencyclidine

Schifano et al., 2005), harms associated with injecting drugs (Hope et al., 2016), and the possibility to develop psychiatric symptoms (Martinotti et al., 2014; Martinotti et al., 2015; Bersani et al., 2014). Beside acute toxicological effects, many NPS seem to have addictive properties (Miliano et al., 2016). In addition to the "classical" NPS, which are classified into known classes of compounds (i.e., cathinones, cannabinoids, phenethylamines, opioids, tryptamines, benzodiazepines, and dissociative anesthetics), law enforcements carry out seizures of compounds that are not classified into these groups of molecules and which are labeled as "other substances" (EMCDDA, 2016). In view of this grows the urgency to understand the pharmaco-toxicological effects of these "other substances" and getting to know their use among consumers. Among these NPS notified to the EMCDDA and Europol, for the first time in 2012 was 1-cyclohexyl-x-methoxybenzene, a molecule that shares some structural characteristics with phencyclidine (PCP) and tramadol although, lacking the amine functionality (Figure 1; EMCDDA, 2012). There has been growing clinical, public, and media awareness and concern about the availability and potential harmfulness of tramadol and PCP. The first one, tramadol, is an atypical, centrally acting synthetic analgesic used to treat moderate to severe pain, with antinociceptive effects that are mediated by a combination of mu opioid agonist effects and norepinephrine (NE) and serotonin reuptake inhibition (Lanier et al., 2010). On the other hand, PCP is a potent hallucinogenic drug, representing a synthetic arylcyclohexylamine originally developed as an anesthetic that acts as a glutamatergic N-methyl-D-aspartate antagonist and also showing cholinergic and monoaminergic activity (Kyzar et al., 2012). Both these drugs are abused (Simonsen et al., 2015; Baumeister, Tojo, and Tracy 2015; Awadalla & Salah-Eldin, 2016) and produced severe adverse effects, characterized by sensory changes, with dissociative, out-of-body feelings and distorted visual and auditory perceptions; cognitive changes, such as memory impairments, altered perception of time, and slowness; affective changes, although quite labile, varying between euphoric, anxious, apathetic, and irritable; unpredictable behavioural changes, potentially including aggression; and changes in consciousness. Moreover, there are considerable risks associated with use, including pulmonary oedema, cerebrovascular accidents, and cardiac arrest (Baumeister et al., 2015; Ryan & Isbister, 2015).

It is important to underline that the "street name" of 1-cyclohexyl-x-methoxybenzene has yet to be discovered. This is highly significant to the understanding of the potential impact of this substance on the market. In fact, there are numerous brand names that identified the NPS (Corazza et al., 2014).

1-cyclohexyl-x-methoxybenzene, seized in Austria by law enforcement as white powder form, has not been stereochemically analyzed, and therefore, it was not possible to know if the seized substance was a single isomer or a mixture of two or all three isomers (*ortho*, *meta* and *para*). This aspect is very important because the different replacements of methoxy group on the benzyl ring (i.e., *ortho*, *meta*, and *para*) may confer different pharmacological and toxicological properties to the molecule. In fact, as reported for other NPS (synthetic cannabinoids), the substitution of a hydroxyl group in ortho, meta, or para position on the benzene ring of the naphthoylindole structure determines a change in the pharmacodynamic properties and biological activity of compounds (Brents et al., 2012; Wiley, Marusich, and Huffman 2014). Nowadays, there is no information on the pharmaco-toxicological effects evoked by 1-cyclohexyl-x-methoxybenzene neither in animal studies and human reports from emergency rooms. Moreover, these NPS are not under legislative control in the world.

Therefore, in this study, the first step was to have synthesized pure and isolated 1-cyclohexyl-x-methoxybenzene derivatives (*ortho*, *meta*, and *para* isomers). Second, because there is an existence of three different isomers of 1-cyclohexyl-x-methoxybenzene, all three of these derivatives were studied to better understand the behavioral effects evoked by each one and their possible comparison. For this purpose, we used a selected battery of behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin 1968; Mattsson, Spencer, and Albee 1996; Porsolt et al., 2002; Redfern et al., 2005; Hamdam et al., 2013; ICHS7A, 2001). From this behavioral test, we evaluated the acute effects of 1-cyclohexyl-x-methoxybenzene isomers (*ortho*, *meta*, and *para*) on sensorimotor responses to visual stimulation, body temperature, acute thermal analgesia, and motor activity in CD-1 male mice.

2 | MATERIAL AND METHODS

2.1 | Animals

Outbred albino male (CD-1®) mice, 25–30 g (Harlan Italy; S. Pietro al Natisone, Italy), were group-housed (8 to 10 mice per cage; floor area per animal was 80 cm^2; minimum enclosure height was 12 cm) on a 12:12-h light–dark cycle (light period from 6:30 AM to 6:30 PM), temperature of 20–22°C, humidity of 45–55% and were provided with ad libitum access to food (Diet 4RF25 GLP; Mucedola, Settimo Milanese, Milan, Italy) and water. The experimental protocols performed in thi study were in accordance with the U.K. Animals (Scientific Procedures) Act, 1986, and associated guidelines and the new European Communities Council Directive of September 2010 (2010/63/EU) a revision of the Directive 86/609/EEC. Moreover, experimental protocols were approved by Italian Ministry of Health and by the Ethical Committee of the University of Ferrara. Adequate measures were taken to minimize the number of animals used, their pain, and discomfort.

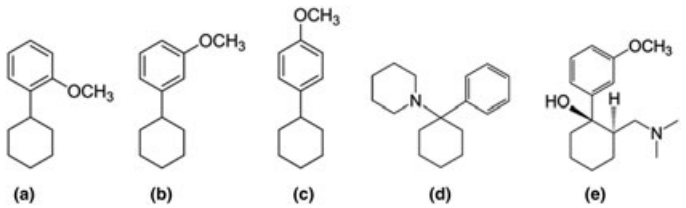

FIGURE 1 Chemical structures of (a) 1-cyclohexyl-2-methoxybenzene (*ortho*), (b) 1-cyclohexyl-3-methoxybenzene (*meta*), (c) 1-cyclohexyl-4-methoxybenzene (*para*), (d) phencyclidine and (e) tramadol

2.2 | Drug preparation and dose selection

1-cyclohexyl-x-methoxybenzene derivatives were synthesized (see Supplementary Materials) and purified (in the laboratory of Dott. Claudio Trapella) with a medium pressure system ISOLERA ONE (Biotage Sweden) and subsequently characterized by Agilent 6520 nano HPLC ESI-Q-TOF (Agilent Technologies) and a Varian 400 MHz NMR. Drugs were initially dissolved in absolute ethanol (final concentration was 2%) and Tween 80 (2%) and brought to the final volume with saline (0.9% NaCl). The solution made with ethanol, Tween 80, and saline was also used as the vehicle. Drugs were administered by intraperitoneal injection at a volume of 4 uL/g. The wide range of doses of 1-cyclohexyl-x-methoxybenzene derivatives tested (0.1–100 mg/kg i.p.) were chosen based on previous safety pharmacology studies on NPS (Vigolo et al., 2015; Ossato et al., 2015, 2016).

2.3 | Behavioral studies

The effect of 1-cyclohexyl-x-methoxybenzene derivatives were investigated using behavioral tests widely used in studies of "safety-pharmacology" for the preclinical characterization of new molecules in rodents (Irwin 1968; Mattsson et al., 1996; Porsolt et al., 2002; Redfern et al., 2005; Hamdam et al., 2013; ICH S7A, 2001). These tests have also been validated to describe effects of cannabinoids on the "tetrad" and sensorimotor changes in mice (Compton et al., 1992; Vigolo et al., 2015; Ossato et al., 2015, 2016).

Behavioral tests were conducted in a thermostated (temperature 20–22°C, humidity about 45–55%) and light controlled (about 150 lux) room in which there was a background noise of about 40 ± 4 dB. The mice were evaluated in functional observational behavioral tests carried out in a consecutive manner according to the following time scheme: observation of visual placing response, measures of core (rectal measurement) body temperature, determination of thermal (tail withdrawal) acute pain, and stimulated motor activity (accelerod test). All experiments were performed from 8:30 AM to 2:00 PM. Experiments were conducted blindly by trained observers working together in pairs (Redfern et al., 2005). The behavior of the mice (sensorimotor responses) were videotaped and analyzed off-line by a different trained operator that would give the test scores.

2.3.1 | Sensorimotor study. *Evaluation of the visual response*

The mice's visual responses were verified by *visual object response* test, which evaluated the ability of the animal to capture visual information even when the animals were stationary. This test was used to evaluate the ability of the mice to see an object approaching from the front (frontal view) or the side (lateral view), then inducing the animals to shift or turn the head, bring the forelimbs into the position of "defence" or retreat from it. For the frontal visual response, a white horizontal bar was moved frontally to the mouse heads and the manoeuvre was repeated three times. For the lateral visual response, a small dentist's mirror was moved into the mice's field of view in a horizontal arc, until the stimulus was between the mice's eyes. The procedure was conducted bilaterally (Ossato et al., 2015, 2016) and was repeated three times. The score assigned was a value of 1 if there was a reflection in the mouse movement, or 0 if not. The total value was calculated by adding the scores obtained in the frontal with that obtained in the lateral visual object responses (overall score 9). Evaluation of the visual object responces were measured at 0, 10, 30, 60, 120, 180, 240, and 300 min post injection. Each mouse was housed in an experimental chamber (350 × 350 × 350 [hr] mm), which was made with black methacrylate walls and a transparent front door. At the top and/or side of the box was placed a camera (B/W USB Camera day & night with varifocal lens; Ugo Basile, Italy). Before the experimental sessions, each mouse was placed in the box and was handled and trained for every other day (once a day) for a week (three days of training in total) in order to get used to the environment and to the experimenter (Ossato et al., 2016). To avoid mice olfactory cues, cages were carefully cleaned with a dilute (5%) ethanol solution and washed with water.

2.3.2 | Evaluation of core body temperature

To better assess the effects of the ligands on thermoregulation, we measured changes in the core (rectal) temperature. Rectal body temperature was used as an index of total body heat at various times during the experiment. The core temperature was evaluated by a probe (1 mm diameter) that was gently inserted, after lubrication with liquid vaseline, into the rectum of the mouse (to about 2 cm) and left in position until the stabilization of the temperature (about 10 s; Vigolo et al., 2015). The probe was connected to a Cole Parmer digital thermometer, model 8402. Stress was equalized to a normal routine clinical procedure. Core (rectal) mouse body temperatures were measured at 0, 30, 50, 85, 140, 200, 260, and 320 min post injection.

2.3.3 | Evaluation of pain induced by a thermal stimulus

Acute thermal nociception was evaluated using the tail withdrawal test (Vigolo et al., 2015). Mice were restrained in a dark plastic cylinder (3 cm long and 6.3 cm diameter) closed at the sides with plastic mesh, which allowed the mice to breathe normally. Then half of the tail was dipped in water of 48°C, and the latency (in seconds), or time, that the tail was left in water was recorded. A cut off (15 s) was set to avoid tissue damage. No signs of damage, burn or variation in mice's tail sensitivity were observed after the repetition of three consecutive tests at 48°C. Acute thermal nociception was measured at 35, 55, 90, 145, 205, 265, and 325 min post injection.

2.3.4 | Motor activity assessment

Alterations of motor activity induced by 1-cyclohexyl-x-methoxybenzene derivatives were evaluated using a behavioral test validated to specifically assess motor behavior (Marti et al., 2004, 2005; Vigolo et al., 2015; Ossato et al., 2016) in dynamic conditions (accelerod test).

The accelerod test measures different motor parameters such as motor coordination, locomotive ability (akinesia/bradykinesia), balance ability, muscular tone, and motivation to run. The animals were placed on a rotating cylinder that increases velocity automatically in a constant manner (0–60 rotations/min in 5 min). The time spent on the

cylinder was also measured. The accelerod test was performed at 0, 40, 60, 95, 150, 210, 270, and 330 min post injection.

2.4 | Data and statistical analysis

The data is expressed in arbitrary units (visual objects response), Δ°C (core temperature, as the difference between control temperature (before injection) and temperature following drug administration), Emax % (tail withdrawal test, calculated as percent of maximal possible effect {EMax% = [(test − control latency)/(cut off time − control)] × 100}) and percentage of basal (accelerod test). All of the numerical data is given as mean ± SEM. The data was analyzed by utilizing repeated measures ANOVA. Results from treatments showing significant overall changes were subjected to *post hoc* Tukey tests with significance for $p < 0.05$.

The statistical analysis of the effects of the individual substances in different concentrations over time was performed by two-way ANOVA followed by Bonferroni's test for multiple comparisons. The analysis of the total average effect induced by treatments (expressed in the panels d) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. The statistical analysis was performed with the program Prism software (GraphPad Prism, USA).

3 | RESULTS

3.1 | Behavioral studies

3.1.1 | Sensorimotor studies. *Evaluation of the visual object response*

Visual object response tended to be reduced in vehicle-treated mice over the 5 hr of observation (~20% of reduction at 300 min; Figure 2a–c), and the effect of which was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives (0.1–100 mg/kg i.p.) reduced in a dose dependent manner the visual object response in mice and the effect persisted up to 5 hr at higher doses (Figure 2a: significant effect of treatment [$F_{4,280} = 27.59$, $p < 0.0001$], time [$F_{7,280} = 15.53$, $p < 0.0001$], and time x treatment interaction [$F_{28,280} = 138.6$, $p < 0.0001$]. Figure 2b: significant effect of treatment ($F_{4,280} = 602.8$,

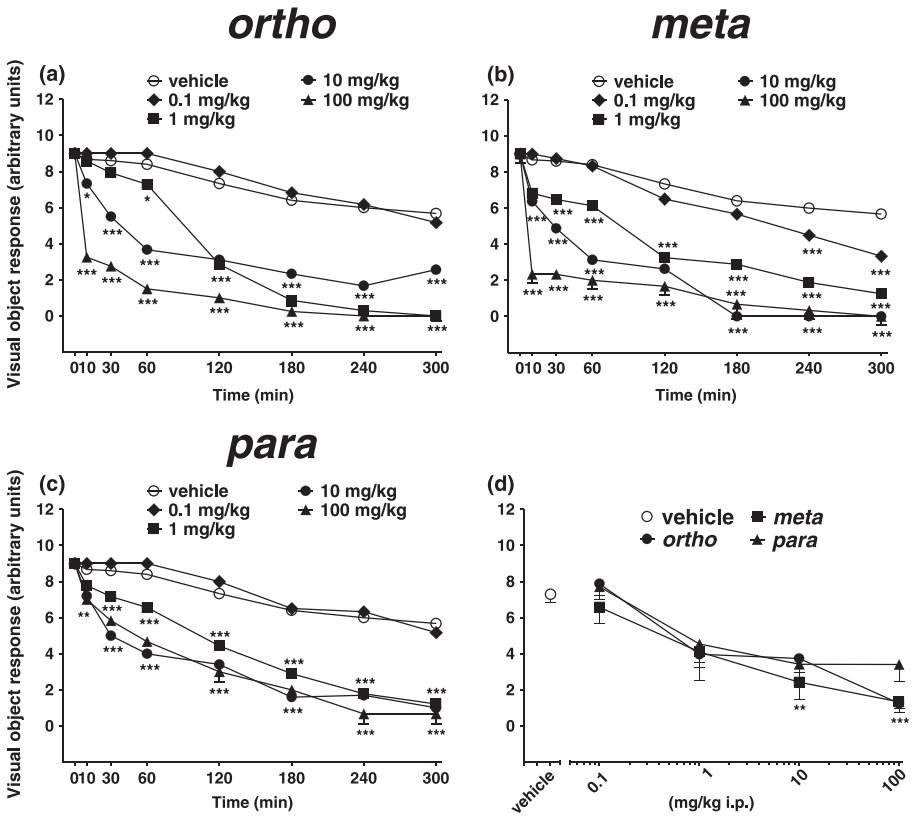

FIGURE 2 Effect of the systemic administration (0.1–100 mg/kg i.p.) of 1-cyclohexyl-2-methoxybenzene (*ortho*; a), 1-cyclohexyl-3-methoxybenzene (*meta*; b), and 1-cyclohexyl-4-methoxybenzene (*para*; c) on the visual object test in mice. Comparison of the total average effect observed in 5 hr (d). Data are expressed (see 2) as arbitrary units and represent the mean ± SEM of eitht determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (a–c), and the statistical analysis of the comparison of the total average effect of the compounds (d) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. (a) Significant effect of treatment ($F_{4,280} = 27.59$, $p < 0.0001$), time ($F_{7,280} = 15.53$, $p < 0.0001$), and time x treatment interaction ($F_{28,280} = 138.6$, $p < 0.0001$). (b) Significant effect of treatment ($F_{4,280} = 602.8$, $p < 0.0001$), time ($F_{7,280} = 416.4$, $p < 0.0001$) and time x treatment interaction ($F_{28,280} = 20.66$, $p < 0.0001$). (c) Significant effect of treatment ($F_{4,280} = 768.00$, $p < 0.0001$), time ($F_{7,280} = 734.2$, $p < 0.0001$), and time x treatment interaction ($F_{28,280} = 30.20$, $p < 0.0001$). (d) 1-cyclohexyl-x-methoxybenzene derivatives ($F_{12,103} = 7.221$, $p < 0.0001$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle

$p < 0.0001$), time [$F_{7,280} = 416.4$, $p < 0.0001$], and time x treatment interaction [$F_{28,280} = 20.66$, $p < 0.0001$]. Figure 2c: significant effect of treatment [$F_{4,280} = 768.00$, $p < 0.0001$], time [$F_{7,280} = 734.2$, $p < 0.0001$], and time x treatment interaction [$F_{28,280} = 30.20$, $p < 0.0001$]). Moreover, the effect induced by *ortho* and *meta* derivatives at 100 mg/kg seems to be earlier than that induced by 1-cyclohexyl-*para*-methoxybenzene at the same dose. The inhibitory effect caused by 1-cyclohexyl-*meta*-methoxybenzene appeared to be more potent than that induced by the other derivatives (Figure 2 d; [$F_{12,103} = 7.221$, $p < 0.0001$]).

3.1.2 | Evaluation of the core body temperature

The core temperature of mice tends to be reduced in vehicle-treated over the 5 hr of observation (~1°C of reduction at 300 min; Figure 3a–c), and the effect was similar to that observed in naïve untreated animals (data not shown). Systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives (0.1–100 mg/kg i.p.) transiently alter the core temperature in mice.

Moreover, although para stereoisomer evoked only a transient moderate reduction in core temperature at 100 mg/kg (approximately −3°C at 30 min time point; Figure 3c; significant effect of treatment [$F_{4,245} = 38.94$, $p < 0.0001$], time [$F_{6,245} = 1.701$, $p = 0.1212$], and time x treatment interaction [$F_{24,245} = 1.374$, $p = 0.1195$]), ortho and meta derivatives induced a biphasic effect (Figure 3a: significant effect of treatment [$F_{4,245} = 21.258$, $p < 0.0001$], time [$F_{6,245} = 1.441$, $p = 0.1995$], and time x treatment interaction [$F_{24,245} = 1.205$, $p = 0.2375$]; and Figure 3b: significant effect of treatment [$F_{4,245} = 28.19$, $p < 0.0001$], time [$F_{6,245} = 0.8032$, $p = 0.5683$], and time x treatment interaction [$F_{24,245} = 0.5357$, $p = 0.9647$]).

In particular, injection of *ortho* and *meta* derivatives at low dose (0.1 mg/kg) induced a transient mild hyperthermia (~1°C at 30 min time point for both drugs; Figure 3a,b). Nevertheless, at the highest dose tested (100 mg/kg), 1-cyclohexyl-*ortho*-methoxybenzene evoked a mild hypothermia during the first 30 min after administration (approximately −1°C Figure 3a), and 1-cyclohexyl-*meta*-methoxybenzene induced a moderate hypothermia in the same time point (approximately −3°C at 30 min time point; Figure 3b).

3.1.3 | Evaluation of pain induced by a thermal stimulus

Systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives (0.1–100 mg/kg i.p.) increased the threshold to acute thermal pain stimulus in mice in the tail withdrawal test ([Figure 4a: significant

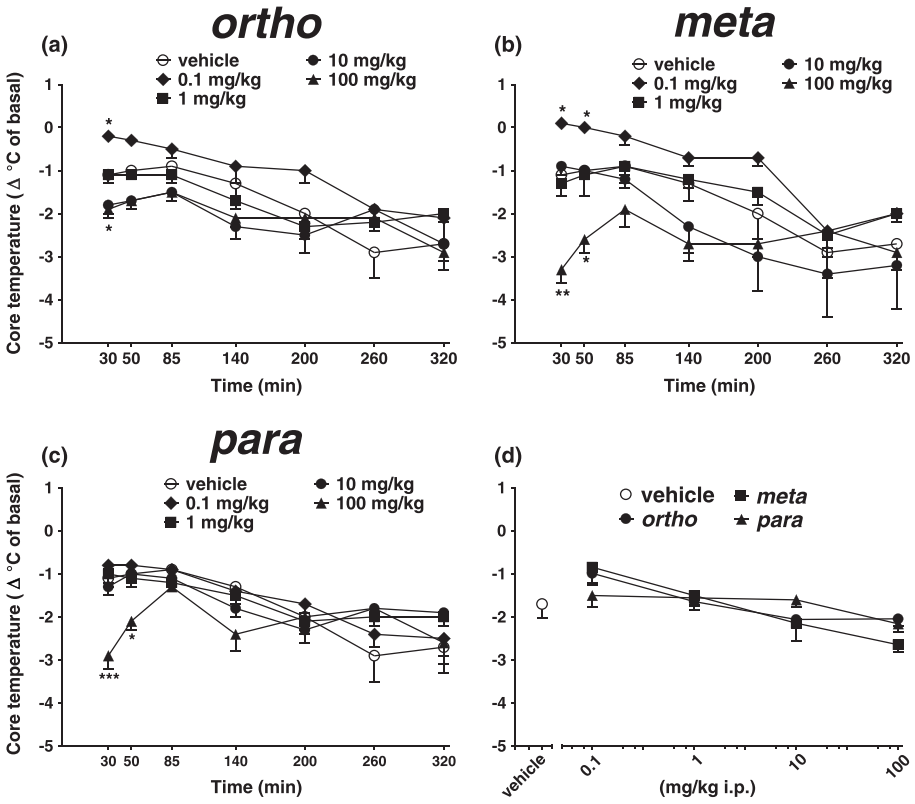

FIGURE 3 Effect of the systemic administration (0.1–100 mg/kg i.p.) of 1-cyclohexyl-2-methoxybenzene (*ortho*; a), 1-cyclohexyl-3-methoxybenzene (*meta*; b), and 1-cyclohexyl-4-methoxybenzene (*para*; c) on mouse core temperature. Comparison of the total average effect observed in 5 hr (d). Data are expressed as the difference between control temperature (before injection) and temperature following drug administration (Δ°C; see 2) and represent the mean ± SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (a–c), and the statistical analysis of the comparison of the total average effect of the compounds (d) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. (a) Significant effect of treatment ($F_{4,245} = 13.33$, $p < 0.0001$), time ($F_{6,245} = 15.25$, $p < 0.0001$), and time x treatment interaction ($F_{24,245} = 1.1$, $p = 0.3440$). (b) Significant effect of treatment ($F_{4,245} = 16.27$, $p < 0.0001$), time ($F_{6,245} = 11.67$, $p < 0.0001$), and time x treatment interaction ($F_{24,245} = 1.343$, $p = 0.1368$). (c) Significant effect of treatment ($F_{4,245} = 5.062$, $p = 0.0006$), time ($F_{6,245} = 12.56$, $p < 0.0001$), and time x treatment interaction ($F_{24,245} = 1.940$, $p = 0.0067$). (d) 1-cyclohexyl-x-methoxybenzene derivatives ($F_{12,103} = 3.723$, $p = 0.0001$). $^{**}p < 0.01$, $^{***}p < 0.001$ versus vehicle

effect of treatment [$F_{4,245}$ = 21.258, p < 0.0001], time [$F_{6,245}$ = 1.441, p = 0.1995], and time x treatment interaction [$F_{24,245}$ = 1.205, p = 0.2375]. Figure 4b: significant effect of treatment [$F_{4,245}$ = 28.19, p < 0.0001], time [$F_{6,245}$ = 0.8032, p = 0.5683], and time x treatment interaction [$F_{24,245}$ = 0.5357, p = 0.9647]. Figure 4c: significant effect of treatment [$F_{4,245}$ = 38.94, p < 0.0001], time [$F_{6,245}$ = 1.701, p = 0.1212], and time x treatment interaction [$F_{24,245}$ = 1.374, p = 0.1195]). In particular, administration of *ortho*, *meta*, and *para* derivatives at a dose of 10 mg/kg induced a long lasting analgesic effect up to the end of the experimental observation.

Furthermore, only *para* derivative at the higher dose (100 mg/kg) increased the threshold to acute thermal pain stimulus (Figure 4c: significant effect of treatment [$F_{4,245}$ = 38.94, p < 0.0001], time [$F_{6,245}$ = 1.701, p = 0.1212], and time x treatment interaction [$F_{24,245}$ = 1.374, p = 0.1195]), and the *ortho* and *meta* derivatives lose their analgesic activity (Figure 4d: 1-cyclohexyl-x-methoxybenzene derivatives [$F_{12,103}$ = 21.97, p < 0.0001]).

3.1.4 | Accelerod test

Systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives (0.1–100 mg/kg i.p.) did not change the motor activity in the accelerod test in mice (Figure 5a: significant effect of treatment [$F_{4,280}$ = 6.781, p < 0.0001], time [$F_{7,280}$ = 0.1394, p = 0.9951], and time x treatment interaction [$F_{28,280}$ = 0.1639, p = 1.00]. Figure 5b: significant effect of treatment [$F_{4,280}$ = 6.075, p = 0.0001], time [$F_{7,280}$ = 0.1622, p = 0.9922], and time x treatment interaction [$F_{28,280}$ = 0.3637, p = 0.9989]. Figure 5c: significant effect of treatment [$F_{4,280}$ = 2.392, p = 0.0509], time [$F_{7,280}$ = 0.2697, p = 0.9653], and time x treatment interaction [$F_{28,280}$ = 0.4221, p = 0.9961]. Figure 5d: significant effect of 1-cyclohexyl-x-methoxybenzene derivatives [$F_{12,103}$ = 17.12, p < 0.0001]).

4 | DISCUSSION

For the first time, this study demonstrates that systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives (*ortho*, *meta*, and *para*) impairs the visual sensorimotor response, the thermal analgesia and modulates core temperature without affecting motor performance on the accelerod test in CD-1 male mice. All three molecules are pharmacologically active with a similar profile of action that slightly differs on core temperature (Figure 3) and thermal pain (tail withdrawal test;

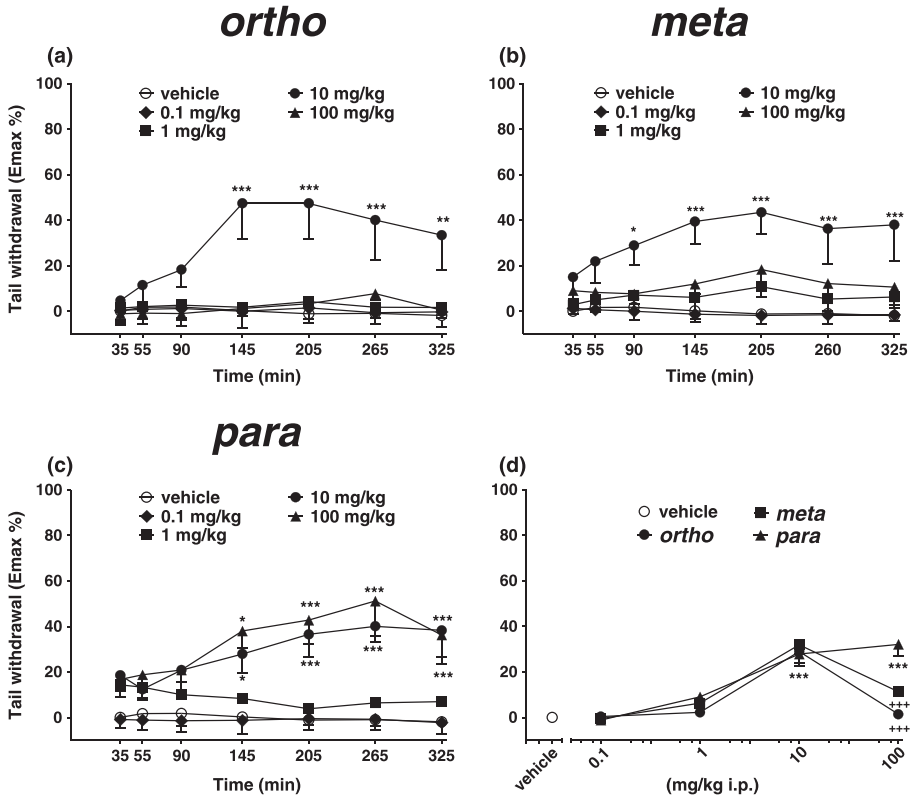

FIGURE 4 Effect of the systemic administration (0.1–100 mg/kg i.p.) of 1-cyclohexyl-2-methoxybenzene (*ortho*; a), 1-cyclohexyl-3-methoxybenzene (*meta*; b), and 1-cyclohexyl-4-methoxybenzene (*para*; c) on the tail withdrawal test of the mouse. Comparison of the total average effect observed in 5 hr (d). Data are expressed as percentage of maximum effect (see 2) and represent the mean ± SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (a–c), and the statistical analysis of the comparison of the total average effect of the compounds (d) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. (a) Significant effect of treatment ($F_{4,245}$ = 21.258, p < 0.0001), time ($F_{6,245}$ = 1.441, p = 0.1995), and time x treatment interaction ($F_{24,245}$ = 1.205, p = 0.2375). (b) Significant effect of treatment ($F_{4,245}$ = 28.19, p < 0.0001), time ($F_{6,245}$ = 0.8032, p = 0.5683), and time x treatment interaction ($F_{24,245}$ = 0.5357, p = 0.9647). (c) Significant effect of treatment ($F_{4,245}$ = 38.94, p < 0.0001), time ($F_{6,245}$ = 1.701, p = 0.1212), and time x treatment interaction ($F_{24,245}$ = 1.374, p = 0.1195). (d) 1-cyclohexyl-x-methoxybenzene derivatives ($F_{12,103}$ = 21.97, p < 0.0001). *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle; ++ +p < 0.001 versus 1-cyclohexyl-4-methoxybenzene

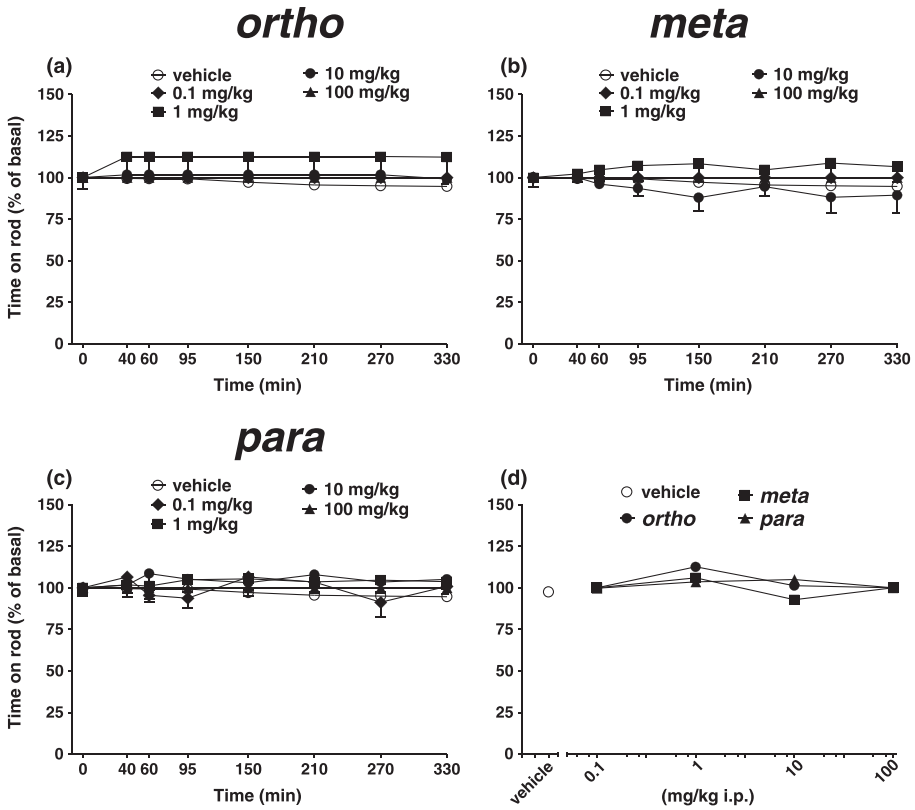

FIGURE 5 Effect of the systemic administration (0.1–100 mg/kg i.p.) of 1-cyclohexyl-2-methoxybenzene (*ortho*; a), 1-cyclohexyl-3-methoxybenzene (*meta*; b), and 1-cyclohexyl-4-methoxybenzene (*para*; c) on the accelerod test of the mouse. Comparison of the total average effect observed in 5 hr (d). Data are expressed (see 2) as percentage of baseline and represent the mean ± SEM of eight determinations for each treatment. Statistical analysis was performed by two-way ANOVA followed by the Bonferroni's test for multiple comparisons for both the dose response curve of each compounds at different times (a–c), and the statistical analysis of the comparison of the total average effect of the compounds (d) was performed with one-way ANOVA followed by Tukey's test for multiple comparisons. (a) Significant effect of treatment ($F_{4,280} = 6.781$, $p < 0.0001$), time ($F_{7,280} = 0.1394$, $p = 0.9951$), and time x treatment interaction ($F_{28,280} = 0.1639$, $p = 1.00$). (b) Significant effect of treatment ($F_{4,280} = 6.075$, $p = 0.0001$), time ($F_{7,280} = 0.1622$, $p = 0.9922$), and time x treatment interaction ($F_{28,280} = 0.3637$, $p = 0.9989$). (c) Significant effect of treatment ($F_{4,280} = 2.392$, $p = 0.0509$), time ($F_{7,280} = 0.2697$, $p = 0.9653$), and time x treatment interaction ($F_{28,280} = 0.4221$, $p = 0.9961$). (d) significant effect of 1-cyclohexyl-x-methoxybenzene derivatives ($F_{12,103} = 17.12$, $p < 0.0001$). ***$p < 0.001$ versus vehicle; °°°$p < 0.001$ versus 1-cyclohexyl-3-methoxybenzene; +++$p < 0.001$ versus 1-cyclohexyl-4-methoxybenzene

Figure 4) modulation probably due to their different substitution on the benzyl ring of the methoxylic group. In particular, systemic administration of 1-cyclohexyl-*ortho*-methoxybenzene and 1-cyclohexyl-*meta*-methoxybenzene evokes a mild core hyperthermia at the lower dose (0.1 mg/kg) and a transient hypothermia at the higher dose (100 mg/kg), and the *meta* derivate seems to be more effective than the *ortho* compound. Otherwise, the 1-cyclohexyl-*para*-methoxybenzene only induces transient hypothermia at the higher dose (100 mg/kg). In the tail withdrawal test, the *para* derivate sustained thermal analgesia up to 100 mg/kg dose, and the *ortho* and *meta* derivates were active at 10 mg/kg, but lose their activity at the higher dose (100 mg/kg).

These NPS were notified for the first time in 2012, and they share some structural characteristics with the "dissociative" anesthetic PCP and the analgesic drug tramadol (EMCDDA, 2012).

In particular, the dual modulation caused by *ortho* and *meta* derivatives on mice's body temperature, with low doses producing hyperthermia and higher doses resulting in hypothermia, resembles that induced by both systemic administration of PCP (Hiramatsu, Nabeshima, and Kameyama 1986) and opioid receptor agonists (Chen et al. 2005) in rodents. In fact, high doses of PCP (40 mg/kg) induced hypothermia, and low doses (5 and 10 mg/kg) evoked hyperthermia or had no effect on body temperature (Itoh et al., 1986; Hiramatsu et al., 1986; Bejanian, Pechnick, and George 1990) through a naloxone-dependent mechanism (Hiramatsu et al., 1986). Similarly, the administration of morphine to rats at doses of 4 to 15 mg/kg produces robust hyperthermia, but progressively higher doses induce hypothermia (Geller et al., 1983). Experiments using selective opioid receptor agonists and antagonists reveal that mu opioid receptor activation is responsible for the hyperthermic response to morphine whereas kappa and delta opioid receptor activation mediates the hypothermic effect of morphine (Rawls & Benamar, 2011). However, other mechanisms controlling the thermoregulation in rodents should be considered since the 1-cyclohexyl-x-methoxybenzene compounds resemble the molecular structures of tramadol (EMCDDA, 2012). This analgesic drug induces in humans a slightly hypothermic status by decreasing the precision of thermoregulatory control in addition to reducing the setpoint (De Witte et al., 1998). This effect is possibly due to its complex mechanism of action characterized by inhibition of the neuronal reuptake of

NE and 5-hydroxytryptamine (5-HT), facilitation of 5-HT release, and stimulation of mu opioid receptors (Raffa, 2006). Each of these mechanisms are likely to influence thermoregulatory control even if little experimental evidence is reported for tramadol. In particular, it has been reported that thermal homeostasis involves a balance between heat production and heat dissipation, and NPS affects both aspects of this homeostatic equation. In fact, administration of psychostimulants like MDMA increase cellular metabolic heat output and stress heat dissipation mechanisms with the onset of sweating delayed. This altered thermal regulation inducing acute hyperthermia can include rare fatalities in human (Parrott et al., 2012; Schifano et al., 2003).

Overall this evidence suggests that *ortho*, *meta*, and *para* derivatives could affect thermal homeostasis in mice possibly through opioid/NE/5-HT receptor mechanisms. Further studies using selective receptor antagonists will be undertaken to better investigate this hypothesis.

Moreover, systemic administration of 1-cyclohexyl-x-methoxybenzene derivatives significantly increases the threshold to acute thermal pain stimulus in mice at the 10 mg/kg dose (Emax ~40%). However, the *ortho* and *meta* isomers lose their analgesic activity at higher dose (100 mg/kg), and the *para* isomer was effective up to 100 mg/kg (Figure 4a–d). The analgesic profile induced by the 1-cyclohexyl-x-methoxybenzene derivates in mice (Figure 4) was similar to that evoked by tramadol in rats and mice (Raffa, 2006; Ozdogan, Lahdesmaki, and Scheinin 2006; Zhang et al., 2011; Aydin, Ek, and Temocin 2012). Because the analgesic actions of tramadol arise from agonist activity of the drug at the μ-opioid receptor and the blockade of serotonin and NE uptake (Raffa, 2006; Andurkar, Gendler, and Gulati 2012), we can hypothesize that these NPS had a similar mechanism of action with tramadol, but with different pharmacokinetic. However, PCP-like mechanisms should be considered also in the analgesic effect of 1-cyclohexyl-x-methoxybenzene derivatives. In fact, although the PCP does not possess an analgesic action to the thermal stimulus, it was reported that methyl substitution on *para* position of the phenyl ring of PCP structure generates a strong analgesic effect (Ahmadi et al., 2011).

It is interesting to note that 1-cyclohexyl-x-methoxybenzene derivatives did not impair Rotarod test performance (Figure 5) as well as tramadol (Ozdogan et al., 2006) but differently by PCP. In fact, PCP caused dose-dependent increases in locomotor activity, assessed in rodents as well as increases in horizontal locomotion, vertical movements such as rearing, and/or stereotypies (Benneyworth, Basu, and Coyle 2011).

Interestingly, these NPS (1-cyclohexyl-x-methoxybenzene derivatives) induced a deep dose-dependent impairment of visual sensorimotor responses in mice during a wide range of doses (0.1–100/kg) that did not cause catalepsy (data not shown), or reduced stimulated motor activity (accelerod test Figure 5). Therefore, these findings point out that effects induced by 1-cyclohexyl-x-methoxybenzene derivatives on visual responses do not result from a disruption of motor function as previously demonstrated also for synthetic cannabinoids (Ossato et al., 2015, 2016). These effects were observed at lower doses that did not affect others behavioral and physiological parameters, suggesting that these compounds primarily induce visual sensorimotor alterations, as caused by the hallucinogenic drug PCP (Morris & Wallach, 2014).

In fact, differently from tramadol, recent evidence show that administration of PCP impairs visual attention in rodents (Varvel et al., 2001; Stefani & Moghaddam, 2002; Terry et al., 2002; Jentsch & Anzivino, 2004), and decreases accuracy in a task designed to evaluate visual perceptual ability at lower doses, but not at the higher ones (Talpos et al., 2015).

5 | CONCLUSION

For the first time, this study reports the chemoselective synthesis of 1-cyclohexyl-x-methoxybenzene derivatives and their in vivo pharmacological effects in CD-1 male mice. This data reveals that these NPS markedly inhibit visual response, promote analgesia and cause core temperature alterations in mice showing a pharmaco-toxicological profile similar to that of PCP and tramadol and suggests their possible dangerousness potential for human health (i.e., hyperthermia and sensorimotor alterations). Although obtained in animal model, this data reinforces the hypothesis that these NPS may have a negative impact in many daily activities, greatly increasing the risk factors for workplace accidents and traffic injuries.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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