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The impact of scopolamine pretreatment on 3-iodothyronamine (T1AM) effects on memory and pain in mice☆

Annunziatina Laurino, Ersilia Lucenteforte, Gaetano De Siena, Laura Raimondi *

Dept. of Neuroscience, Drug Sciences, Psychology, Child Health and Pharmacology, University of Florence, Viale G. Pieraccini, 6, 50139 Florence, Italy

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ABSTRACT

We previously demonstrated that 3-iodothyronamine (T1AM), a by-product of thyroid hormone metabolism, pharmacologically administered to mice acutely stimulated learning and memory acquisition and provided hyperalgesia with a mechanism which remains to be defined. We now aimed to investigate whether the T1AM effect on memory and pain was maintained in mice pre-treated with scopolamine, a non-selective muscarinic antagonist expected to induce amnesia and, possibly, hyperalgesia.

Mice were pre-treated with scopolamine and, after 20 min, injected intracerebroventricularly (i.c.v.) with T1AM (0.13, 0.4, 1.32 μg/kg). 15 min after T1AM injection, the mice learning capacity or their pain threshold were evaluated by the light/dark box and by the hot plate test (51.5 °C) respectively. Experiments in the light/dark box were repeated in mice receiving clorgyline (2.5 mg/kg, i.p.), a monoamine oxidase (MAO) inhibitor administered 10 min before scopolamine (0.3 mg/kg).

Our results demonstrated that 0.3 mg/kg scopolamine induced amnesia without modifying the murine pain threshold. T1AM fully reversed scopolamine-induced amnesia and produced hyperalgesia at a dose as low as 0.13 μg/kg. The T1AM anti-amnestic effect was lost in mice pre-treated with clorgyline.

We report that the removal of muscarinic signalling increases T1AM pro learning and hyperalgesic effectiveness suggesting T1AM as a potential treatment as a "pro-drug" for memory dysfunction in neurodegenerative diseases.

1. Introduction

3-iodothyronamine (T1AM) is a trace amine circulating in mammals which is thought to be a by-product of T3 alternative metabolism (Scanlan et al., 2004). While the physiopathological meaning of T1AM tissue levels is largely unknown, pharmacological evidence suggests that T1AM induces effects similar but also opposite to those elicited by thyroid hormone without interacting at hormone receptors. In fact, T1AM pharmacologically administered at low doses (1.32 and 4 μg/kg i.c.v. injected) quickly modifies murine behavior, including stimulation of learning, memory consolidation, and produces hyperalgesia (Manni et al., 2013). Interestingly, the T1AM pro-learning effect depends on its oxidative deamination producing 3-iodothyroacetic acid (TA1; Musilli et al., 2014) and by the release of histamine (Laurino et al., 2015a). Consistently, TA1 injected i.c.v. but also i.p. stimulates learning and is hyperalgesic, again depending on histamine release (Musilli et al., 2014) (Laurino et al., 2015a).

We also have demonstrated that TA1 accumulates in murine organotypic hippocampal slices exposed to T1AM (Laurino et al., 2015b), thus confirming a possible role for the acid in neuronal signaling.

However, also T1AM has a complex pharmacodynamic profile, being considered a multi-target molecule since amine interaction at several G-protein-coupled receptors including trace amine-associated receptors (TAAR1 and 8; Scanlan et al., 2004; Mühlhaus et al., 2014), α2 (Regard et al., 2007; Dinter et al. 2015a), β2 adrenoreceptors (Dinter et al., 2015b) and ion channels (Lucius et al., 2015) have been reported. Until now, none of these targets has been recognized to be involved in the behavioral effects of low T1AM doses, including memory stimulation and hyperalgesia.

Memory impairments are associated with different and distinctive painful experiences (Kunz et al., 2015), suggesting that memory and pain circuits share common mediators and pathways including muscarinic signaling. Consistently, the pharmacological removal of muscarinic signaling, by using muscarinic antagonists, produces amnesia and hyperalgesia (Khakpai et al., 2012) (Ghelardini et al., 1998).

Whether T1AM effects on memory and pain depend in some way on muscarinic signaling is unknown. To investigate this point, we studied the effect of intracerebroventricular (i.c.v.) injection of T1AM (0.13,

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* Corresponding author.
E-mail address: laura.raimondi@unifi.it (L. Raimondi).

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0.4, 1.32 μg/kg) on the memory and pain threshold of mice pre-treated with scopolamine (0.3 mg/kg).

In addition, since we previously verified that T1AM pro-learning activity was blunted under conditions of systemic MAO inhibition, we repeated the passive avoidance task in mice pre-treated with clorgyline, a monoamine oxidase (MAO) inhibitor.

2. Methods

2.1. Animals

Male mice (CD1 strain; 20–30 g) from the Harlan-Nossan breeding farm were used. The cages were placed in the experimental room 24 h before the tests for adaptation. Animals were kept at 23 ± 1 °C with a 12 h light–dark cycle (light on at 07:00 h) and were fed a standard laboratory diet with water *ad libitum*. Experiments and animal use procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1996). The experimental protocols were approved by the Italian Ministry of Health (n#959, 2015) in compliance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS no. 123) and the European Community Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

2.2. I.c.v. injection technique

I.c.v. administration was performed under light ether anesthesia according to the method described by Haley and McCormick (1957) with minor modifications. The depth of anesthesia was checked by monitoring respiratory rate (which was reduced within 2 min) and testing the lack of pain response to gentle pressure on the hind paws. The head of the anesthetized mouse was grasped firmly and the needle of a 10 μl microsyringe (Hamilton) was inserted perpendicularly 2 mm through the skull into the brain. Ten microliters of solution were then slowly injected (in 20 s) into a lateral ventricle. The injection site was 1 mm to the left of the midpoint, on a line drawn through to the anterior base of the ears. Immediately after needle removal, the animal remained quiet for approximately 1 min and then resumed its normal activity. To ascertain that solutions were administered exactly into the cerebral ventricle, some mice were injected with 10 μl of 1:10 India ink and their brains were examined macroscopically after sectioning. The accuracy of the injection technique was confirmed since 95% of injections turned out to be correct.

2.3. Behavioral tests

2.3.1. The passive avoidance paradigm

The test was performed according to the step-through method described by Manni et al. (2013). The experimental apparatus consisted of a two-compartment acrylic box consisting of an illuminated platform 59–64 m (60 W, 840 lx) connected to a dark chamber by a guillotine door. The dark chamber was constructed with a pitfall floor. When entering this chamber in the training session, mice receive a non-painful punishment consisting of a fall (from 40 cm) into a cold water bath (10 °C). The test was then repeated 1 and 24 h after the training session. Since mice prefer darkness to light, they usually entered the dark compartment within 5 s. Mice not entering the dark compartment within 60 s during the training session were excluded from the experiment. The extent of punishment memory was measured 1 h and 24 h after the training session and expressed as the increase in the latency to enter the dark compartment. In the 1 h and 24 h tests the latency was measured for up to a maximum of 300 s.

Before trials, mice were removed from the cage and treated i.p. with scopolamine (0.3 mg/kg). Mice were then anesthetized as described above and, 15 min after scopolamine were injected i.c.v. with 10 μl 0.5% DMSO (Veh) or T1AM (0.13, 0.4, 1.32 μg/kg) prepared in 0.5% DMSO; (Veh.) n = 10 mice for each treatment.

In another set of experiments, mice also received clorgyline (2.5 mg/kg) 10 min before scopolamine (0.3 mg/kg). 15 min after T1AM injection, mice were placed on the illuminated platform and allowed to enter the dark compartment (training session).

T1AM was kindly provided by Dr. Thomas Scanlan (Portland, OR, USA).

2.3.2. The hot plate

After introducing mice to a hot-plate device (51.5 ± 1 °C), the latency of a flinching or jumping response was measured. The cut-off time was set at 45 s to minimize skin damage.

Anesthetized mice were treated i.p. with 0.3 mg/kg scopolamine or saline and, after 15 min they were injected i.c.v. with vehicle or T1AM (0.13, 0.4, 1.32, and 4 μg/kg, n = 10 in each group). Measurements were performed 15 min after i.c.v. injections.

2.3.3. Statistical analysis

Data were expressed as mean ± SEM of independent experiments. Statistical analysis was performed using one-way or two way ANOVA, followed by the Bonferroni multiple comparison *post hoc* test; the effect size was calculated by the Cohen's d value and by the eta squared. The threshold of statistical significance was set at $P < 0.05$. Data analysis was performed using the GraphPad Prism 5.0 statistical program (GraphPad software, San Diego, CA, USA). When using two-way ANOVA,

3. Results

3.1. T1AM reversed scopolamine-induced amnesia

Before testing the effect of T1AM, we confirmed scopolamine treatment (0.3 mg/kg, i.p.), at our experimental settings, produced amnesia in mice at retention time of 1 and 24 h. Consistently, a two-way ANOVA indicated a significant effect of the treatment (scopolamine) [($F_{(1,36)} = 38.92$, $P < 0.0001$, $\eta^2 = 0.05$), of the time of retention [($F_{(1,36)} = 7.60$, $P = 0.0091$, $\eta^2 = 0.10$)], and no significant ($P > 0.05$) interaction between treatment and time of retention. Mean ± SEM of latency (s) of scopolamine and of Veh.-scopolamine was 34.04 ± 5.7 and 131.60 ± 24.9 ($P < 0.01$, $d = -1.7$), respectively, at 1 h, and 51.56 ± 23.73 and 240.9 ± 30.0 ($P < 0.001$, $d = -2.21$), respectively, at 24 h.

At these conditions of amnesia (Fig. 1, panel A), a two-Way ANOVA indicated a significant effect of adding T1AM treatment [($F_{(3,99)} = 14.23$, $P < 0.00915$, $\eta^2 = 0.18$)], of the retention time [($F_{(2,99)} = 32.72$, $P < 0.0001$, $\eta^2 = 0.30$)], and of interaction between treatment and retention time [($F_{(6,99)} = 3.66$, $P < 0.0025$, $\eta^2 = 0.46$)]. In particular, the mean ± SEM of latency (s) measured for TIAM and scopolamine treated mice was: for 0.14 μg/kg TIAM, 139.50 ± 52.74 and 33.7 ± 4.73 ($P < 0.05$, $d = 1.50$, respectively, at 1 h, and 218 ± 33.70 and 45.61 ± 17.20 ($P < 0.001$, $d = 2.51$), respectively, at 24 h; for 0.4 μg/kg T1AM, 231.66 ± 44.0 and 33.7 ± 4.73 ($P < 0.000$, $d = 3.37$) respectively, at 1 h, and 190 ± 39.11 and 45.61 ± 17.20 ($P < 0.001$, $d = 1.93$), respectively, at 24 h; for 1.32 μg/kg T1AM, 182.75 ± 40.74 and 33.7 ± 4.73 ($P < 0.05$, $d = 1.65$), respectively, at 1 h, and 167.25 ± 36.50 and 45.61 ± 17.20 ($P < 0.001$, $d = 1.30$) respectively at 24 h.

3.2. T1AM reversion of scopolamine amnesia depends on T1AM oxidative metabolism

The light/dark paradigm was then repeated with mice pre-treated i.p. with clorgyline (2.5 mg/kg) and then with scopolamine (0.3 mg/kg). At these conditions, we first verified whether scopolamine treatment maintained an effect on the latency to enter in the dark