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Assessment of Cognitive Deficits Associated with Paint Thinner Inhalation Using Morris Water Maze

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Abstract:	As a global worldwide phenomenon, the intentional inhalation of volatile solvents is associated with devastating health and societal consequences. Cognitive impairments are among the most debilitating medical complications related to the chronic exposure to these substances. Here, we describe an adapted protocol of the Morris Water Maze (MWM) for testing cognitive impairments associated with inhalation of volatile solvents. Originally conceived by Richard G. Morris to assess spatial learning in rats, the MWM has since been used widely in Behavioural Neuroscience to investigate spatial memory-dependent and non-spatial memory dependent neurophysiological functions. The test is relatively simple and takes advantage of the strong motivation that the immersion into water provides for learning in rodents.
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Title:

Assessment of Cognitive Deficits Associated with Paint Thinner Inhalation Using Morris Water Maze

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Keywords:
Neuroscience, Volatile abuse, Thinner inhalation, Memory deficits, Morris Water Maze, Mouse model

Short abstract:
Here, we describe an experimental protocol to investigate cognitive impairments associated with inhalation of volatile solvents using Morris Water Maze in mice as animal model.

Long abstract:
As a global worldwide phenomenon, the intentional inhalation of volatile solvents is associated with devastating health and societal consequences. Cognitive impairments are among the most debilitating medical complications related to the chronic exposure to these substances. Here, we describe an adapted protocol of the Morris Water Maze (MWM) for testing cognitive impairments associated with inhalation of volatile solvents. Originally conceived by Richard G. Morris to assess spatial learning in rats, the MWM has since been used widely in Behavioural Neuroscience to investigate spatial memory-dependent and non-spatial memory dependent neurophysiological functions. The test is relatively simple and takes advantage of the strong motivation that the immersion into water provides for learning in rodents.

Introduction:
Overwhelming evidences from epidemiological studies and neuropsychological tests in solvents abusers indicate that the chronic abuse of inhalant substances lead to learning and memory deficits[1,2,6]. In spite of this, very few animal modelling studies have assessed the neuropathophysiology behind these cognitive impairments[6].

In rodents, a substantial number of behavioural paradigms have been developed[15]. Each test is characterised by specific advantages and inherent disadvantages[15]. In Behavioural Neuroscience, Morris water maze has become, since its development by Richard G. Morris in 1982, the Gold standard behavioural test to assess spatial memory and learning performances in rodents[3,4]. The test (or its many modified versions) has been used in many different fundamental and translational applications[5].

Learning in the MWM has been shown to be faster possibly as a result of the aversive nature, in rodents, of immersion into water[5]. This advantage is also considered a source of stress that has been shown to be associated with neurophysiological changes that could potentially interfere with cognitive assessment[15]. A successful way to overcome this limitation and decrease the level of stress is pre-habituation[8]. Compared to dry-land mazes (such as T-maze, radial maze, complex ally maze) however, MWM does not suffer from the confounding factor of using olfactory cues to orient themselves in the pool. This sets the MWM as an ideal behavioural test for evaluating spatial learning and memory[15]. Recently, the MWM test was successfully used to investigate the cognitive disturbances that are precipitated by inhalation of volatile substances[7]. In this protocol, we describe an adapted version of the test to investigate the acute and chronic effects of paint thinner inhalation on spatial learning and memory function in mice.

Protocol:
All the results described in the current paper are based on the experiments conducted in the Laboratory of Pharmacology, Neurobiology and Behavior in Cadi Ayyad University. All the procedures are therefore in accordance with the Cadi Ayyad University Animal care. The video shootings of the protocol are however performed in LUMC and in strict accordance with LUMC animal Use committee guidelines.

NOTE: The overall timeline of the entire experimental protocol is shown in **Figure 1**.

1. Acute and chronic paint thinner inhalation

Note: The purpose of this protocol is to model solvent abuse in humans using mice. As outlined by Bowen[6], a reliable approach to mimic abuse conditions should include: 1) An inhalation route of exposure, 2) A rapid onset of very high concentrations and 3) Brief periods of exposure. To achieve these conditions, a static system of solvents delivery is used as described below:

1.1. The system is a relatively simple one and consists of a sealed Plexiglas chamber (L:30cm, W:17,5cm, H:15cm) in which a fixed amount of paint thinner is injected onto filter paper within the chamber. Vary the amount of paint thinner injected into the chamber according to the desired final concentration of thinner in the chamber.

Note: This relationship should be calculated in advance using Gas Chromatography to determine the relative density of thinner in air samples taken from the chamber. A detailed description of the methodology used can be found in ref 14. Briefly, a standard linear curve describing the relation between the amount of solvent injected into the chamber and the density of thinner is drawn[14]. In a well-controlled and standard conditions, the slope of the curve will correspond to a conversion factor according to which a determined volume of paint thinner injected into the chamber will correspond to a defined concentration of thinner in the air of the chamber. In our system, the conversion factor was (1 µl ≈ 1.5 ppm).

1.2. One day before the exposure, let the animals (male Swiss mice, n=6 per group) to freely explore the chamber for 30 min. This habituation phase is essential to rule out any novelty-related stress that could potentially interfere with the performance of the animals in the MWM test following thinner exposure.

1.3. Expose the animals to the thinner individually. On the day of exposure, place a mouse in the bottom of the chamber and inject a desired amount of paint thinner into the filter paper and close the chamber.

1.4. Expose for 15 min twice separated by a 5 min interval. Bring the animals back to their home cage during the 5 min interval. At the end of exposure, the animals are ready to perform the MWM behavioural test.

Note: Control mice are subjected to the same procedure except that water, instead of paint thinner, is injected into the filter paper.

1.5. For the chronic regime of paint thinner inhalation, use the same procedure except that the duration of exposure is a 1 hour daily over 45 consecutive days.

Note: The aim of this protocol is to assess the long-term and lasting effect of chronic inhalation of paint thinner on learning and spatial memory. Unlike the acute paradigm, do not start behavioural tests immediately following the last exposure episode. Rather, a period of at least 24 h should separate the last exposure and the behavioural test in order to allow the animals to recover from the acute and short term effects of paint thinner inhalation

2. The Morris Water Maze test

Note: The MWM is a behavioural test widely used to assess spatial memory and learning in rodents[5]. The test takes advantage of the natural aversion of rodents toward immersion into water to provide a strong motivation for learning. Animals use distinct and fixed visual cues to locate a submerged escape platform in a large pool of water. The procedure used for MWM test is based on previously described protocol[8,9].

2.1. Use a large circular pool of about 90 cm in diameter and 35 cm high. Fill the pool with water for which the temperature is maintained within two degrees of 22 °C.

Note: If using black mice, a white background in the pool should be used. If however, if testing white mice, then a black pool should be used. For mice, the diameter of the pool can oscillate between 75 and 150cm[5].

2.1.1. Use a home-made plastic stand with circular top measuring 12 cm in diameter as the escape platform. Submerge the platform 1.5 cm below the surface of the water.

2.2. Place few (3 to 5) distinct visual cues around the pool in the experimental room. Keep the location of these cues close and constant throughout the days of the experiment.

Note: According to our experience, black and white striped objects yield better learning performances.

2.3. Before starting the training phase of the test, administer a cued version of the test on day 1 to all animals of your experimental groups. This test consists of 4 consecutive trials during which the animals learn that the platform is an escape location.

2.3.1. For these trials, while still submerged 1.5 cm below the surface of water, make the platform visible by placing a striped cue above it. This cued version of the test aims to rule out any sensory-motor related differences between the experimental groups before starting the learning phase.

Note: Both the MWM apparatus and the experimental room are now ready to administer the test.

2.4. Performed the MWM test over 9 days that consist of 2 phases; a learning phase of 5 consecutive days and a single day of probing. Separate the two phases by a retention period of 3 days during which the animals are returned and kept in their home cages.

2.5. Expose the animals to a desired concentration of paint thinner (see steps 1.3-1.4).

2.6. To start the learning phase, divide the MWM tank into four virtual equal quadrants. Place the escape platform in one of the four quadrant labelled henceforth the target quadrant. Through the entire learning phase, maintain the platform in the same target quadrant.

2.6.1. Train the animals for four trials per day for 5 consecutive days to navigate towards and locate the platform using distal cues. To prevent the animals from developing egocentric spatial strategies to find the platform, alternate the starting points randomly between each trail.

2.7. Begin a trial by placing the mouse into the water facing the wall of the pool at one of the starting points. Give the mouse 60 s to find the hidden platform. If a mouse failed to locate the platform within this period, gently guide it to the platform and allow it to stay on the platform for 30 s before being returned to their cage.

2.8. On day 9, 72 h after the last learning trial, administer a similar trial as in the learning phase, except that the hidden platform is removed from the pool. Use the preference that the animal has for the target quadrant (as measured by the percentage of time spent swimming in the quadrant) as a measure of spatial memory consolidation. In this trial, do not pre-expose the animals to paint thinner.

2.9. The parameters measured depend on the phase of the test;

2.9.1 For the cued version of the test, Use the latency to find the platform (time separating the start of the trial and the finding of the platform) and the velocity of swimming (Distance travelled while swimming per time unit) as indicators of respectively visual and motor performances. Any differences between the experimental groups in these parameters should be account for in the interpretation of results from the learning and the probe trials.

Note: Among the acute effects of thinner inhalation, sedation induces a significant deterioration of sensory and motor abilities that impact on the performances of the animals in the MWM test[7]. When conducting a MWM test to assess the acute effect of thinner inhalation on spatial memory learning, it is therefore mandatory to precede the learning trials with a cued version of MWM. Only when the sedative effect of thinner inhalation has dissipated that the learning trials could start.

2.9.2 For the learning phase, Use the evolution over days of the escape latencies –defined as the time between the start of a trial and the finding of the hidden platform– as a measure of spatial learning. In normal mice, escape latencies decrease gradually over the 5 learning days to reach less that 10s in the last trials of Day 5 (**Figure 2A,B**).

Note: The shortening of swimming path length over the 5 days of learning phase could also be used as an indication of spatial learning.

2.9.3 During the probe phase, Use the relative time spend by the animals swimming in the target quadrant compared to other quadrant of the maze as a measure of spatial memory consolidation (**Figure 2C**).

Note: All these parameters could be monitored and obtained manually while conducting the essays. If however, video recording is used to film the behavioural tests, then the parameters could be extracted from the videotapes.

Representative results:
By using MWM, recent studies have investigated the cognitive abnormalities induced by acute and chronic inhalation of paint thinner in mice[7,9]. **Figure 2** depicts representative results[9]. The mean escape latencies decreased progressively and significantly over the learning days. After acute inhalation of thinner, the performances of the animals was not affected (**Figure 2A**). This lack of a significant effect of acute inhalation of paint thinner was attributed to the relative low doses of thinner used[9]. Recently Gmaz *et al.* (2012) exposed rats to 30 min of 5,000 ppm of toluene. In this study, 5,000 ppm of toluene induced an acute sedative effect that impacted on sensory-motor abilities of rats during the performance of the MWM test[7]. None of the doses used in our study impaired the sensory-motor performances of the animals.

In contrast to acute exposure to thinner, chronic 1 h daily inhalation of paint thinner for 45 days lead to significant impairment of spatial learning. This is evidenced by a significant increase in escape latencies in 450 and 600 ppm-treated mice compared to controls in day 2 and 3 of the learning phase (**Figure 2B**). The results of the probe trial show that 450 and 600 ppm treated nice spent significantly less time in the target quadrant where the hidden platform was previously located (**Figure 2C**). Taken together, these results indicate that exposure to paint thinner is associated with significant cognitive deficits that could be modelled and investigated using MWM.

Figure Legends:
Figure 1: Schematic representation of the overall timeline of the experimental protocol. Head arrows represent sessions of the acute exposure of paint thinner preceding behavioural tests. When assessing the chronic effects of paint thinner, exposure sessions are performed well before behavioural testing. In this case, the timing of inhalation relative to MWM testing depends on the question investigated.

Figure 2: Representative results of the effect of different concentrations of paint thinner inhalation on the Morris Water Maze test in mice. Chronic (**B**) and not acute (**A**) inhalation induces impairment on the acquisition of spatial learning. (**C**) During the probe trial, 450 and 600ppm-treated mice spend significantly less time swimming in the target quadrant showing an impairment of spatial memory. N=6 mice for each group. *P<0.001 by ANOVA. (A and B have been modified from ref 9).

Discussion:
The deliberate inhalation of volatile substances is a worldwide health issue widespread mainly among adolescents of low income social class[2]. The abuse of these substances is associated with a wide range of neurological and neuropsychological sequels[2,6]. The intentional abuse of solvents should be distinguished from occupational exposures[6]. Abuse patterns involve usually inhalation of high concentrations of solvents over repetitive short periods of time (10-15 min)[6,9]. Occupational exposures in contrast refer to the chronic presence of low levels of inhalants in the work place over long periods of time (6 hours or more)[6].

In an attempt to mimic those two modes of intoxication, animal model studies are generally designed to replicate either the occupational or the abuse pattern of inhalation[6]. Two types

of solvents delivery setups are used in these studies; static and dynamic systems[6]. In static systems, as the one we describe here, animals are placed for a short duration of time within a sealed chamber and a fixed volume of inhalant is injected onto a filter paper. In dynamical delivery systems, a constant concentration of inhalant is delivered through a continuous flow of air connected to the exposure chamber via an inlet and evacuated through an outlet[13].

To model the abuse pattern of solvents inhalation, it is very important to expose animals to repetitive short periods (no more than 15min) of solvents. The rational of using 2 x 15 min separated by 15 min in our protocol is twofold. First, epidemiological studies have revealed that the average duration of repetitive sessions of inhalations among abusers approximates 15 min[10]. The second reason is related to the accumulation of carbon dioxide (CO_2) over time in the inhalation chamber. Reaching a certain threshold, CO_2 could potentially influence the outcome of subsequent behavioural testing. To avoid this, the 30 min duration of inhalation was divided in 2 x 15 min separated by 5 min during which the animals were allowed to breathe fresh air in their home cage. By comparison, and due to the continuous flow of new air in dynamical delivery systems, the possibility of CO_2 accumulation is avoided. This characteristic makes dynamical systems more suitable for modelling occupational, long term exposition to solvents.

While conducting the MWM, few crucial practical points should be considered; 1) If the experimental room is not sound-proof, it is recommended to use 'white noise' as a background to neutralize potential external noises from the surrounding animal facilities that could distract animals from performing behavioural tests, 2) Environmental factors such as light and temperature are potential sources of stress to animals[11] and should therefore be monitored and kept constant throughout the testing, 3) The majority of solvents such as paint thinner induce a constellation of acute complications including sedation and sensory-motor dysfunctions. These alterations do affect the performance of the animals in the MWM[7] and should therefore be taken into account in the protocol designs and interpretation of MWM results, And finally 4) Although the MWM is primarily designed to assess spatial learning and memory[3,4], the test paradigm could be modified to investigate other physiological functions such as working memory, reference memory and task strategy[5].

Such MWM versions are relevant in studying other cognitive impairments associated with solvents abuse. For example Gmaz *et al.* used the reversal version of MWM test to study the cognitive inflexibility precipitated by inhalants[7]. The reversal learning is performed after the learning phase and consists of a trial in which the animals learn to locate the hidden platform after its replacement in a new location. The amount of time spend searching for the platform in its previous location versus its new location is an index of perseveration and cognitive inflexibility[12]. In rats, acute toluene exposure have been found to severely impair reversal learning in the MWM test[7].

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Disclosures:
The authors declare that they have no competing financial interests.

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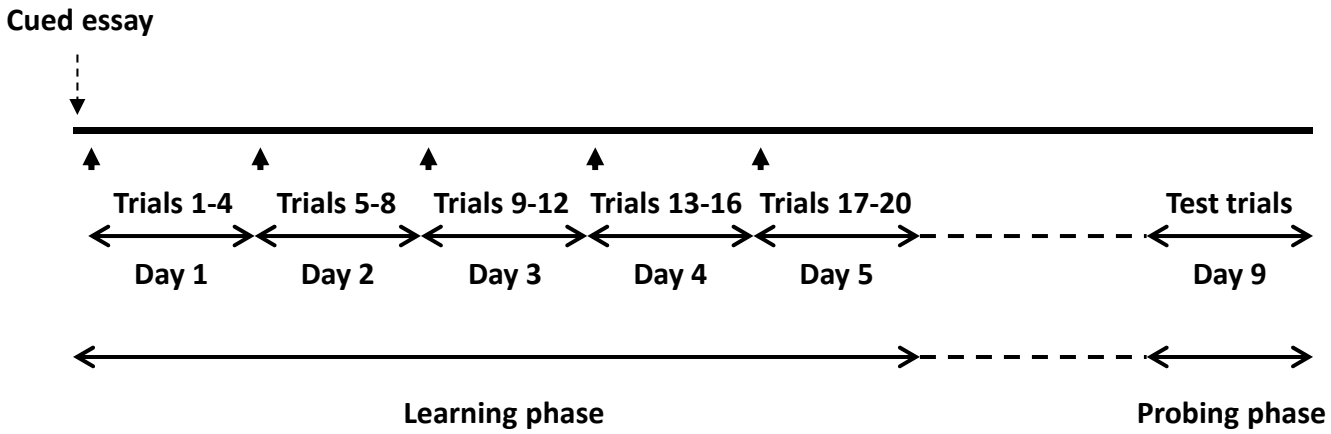

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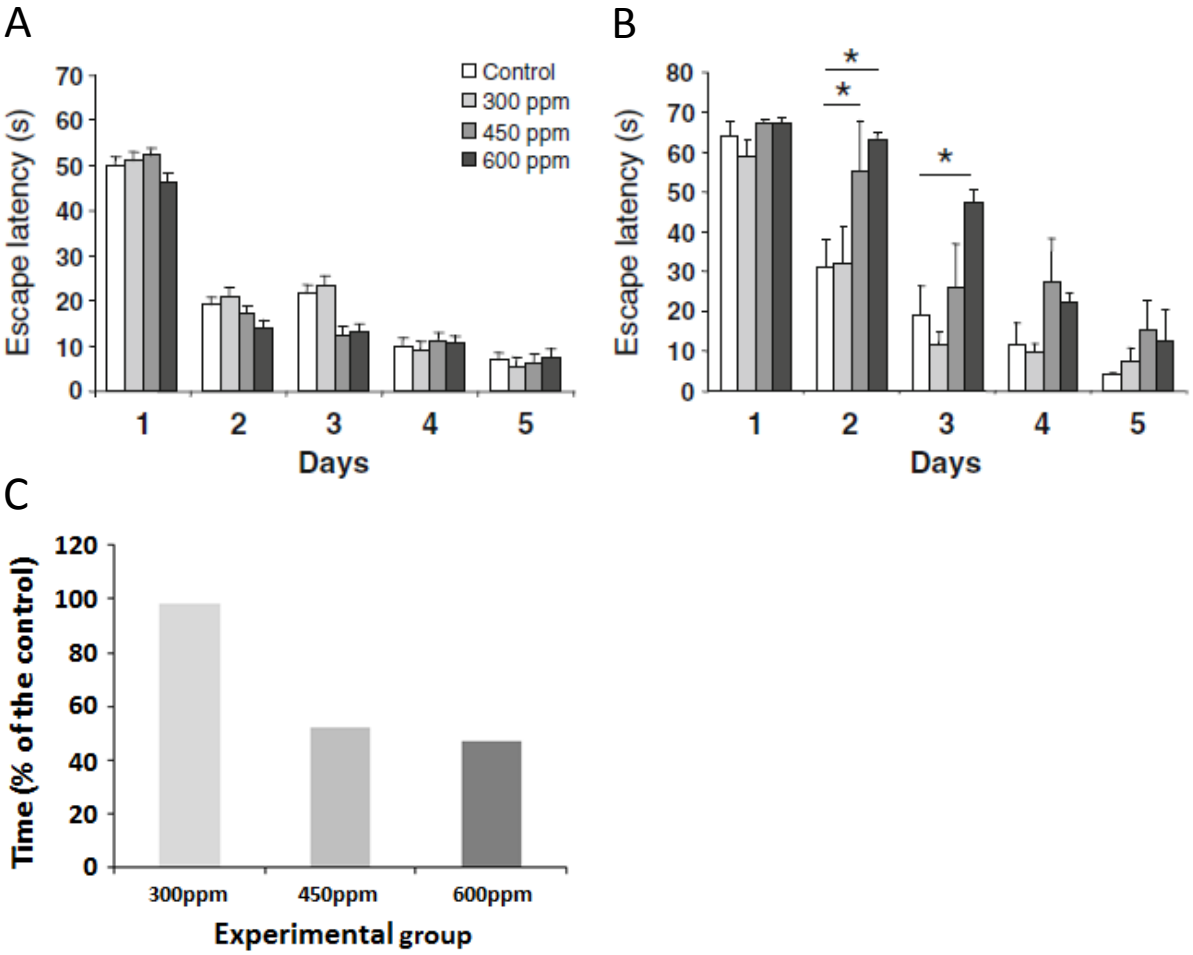

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Paint thinner	Vepec. S.a.r.l. Casablanca, Morocco	None	Paint thinner is a solvent usually used to thin oil-derived paints. Although the chemical composition may differ depending on the manufacturer, toluene remain the main component of the mixture
Filter paper	-	None	Coton could also be used for the same purpose
Water maze pool	Home made	None	None

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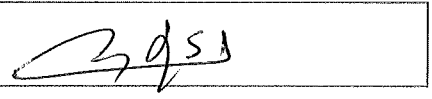
Signature:	
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Second round of responses to the editor and reviewers
Click here to download Rebuttal Comments: Responses to the editor and reviewers.docx

Dear Editor,

We would like to thank you and the reviewers for their constructive remarks of our manuscript entitled '***Assessment of Cognitive Deficits Associated with Paint Thinner Inhalation Using Morris Water Maze***'. By addressing the editorial's comments and those raised by the reviewers, we provide an improved version of the draft and emphasize more explicitly the main steps of our protocol.

NB: all changes in the draft are highlighted in 'Track changes' mode for recognition.

Responses to Editorial comments:

•The manuscript would benefit from proofreading.

We read the final draft at multiple occasions to correct any remaining typos and errors.

•The authors do not have a materials table, which should include paint thinner, filter paper, the pool, any software used for data analysis, and any other purchased equipment.

We added a material table listing all used equipment in this protocol.

•There are some formatting issues which need to be corrected:

-Line 78: Avoid use of personal pronoun "we"

We have removed the personal pronoun and replaced the whole sentence by the following one: 'The purpose of this protocol is to model solvent abuse in humans using mice. …'.

-Why is 2.9 in bold?

This is not anymore in bold characters.

•Additional detail is required in a number of areas:

-2.8: How is this recorded? By hand or by video recording? How is the time spent in the quadrant determined?

We added the following paragraph in page 5 in order to stress the possibility of extracting data either directly while conducting the behavioural tests or from videotapes:
'All these parameters could be monitored and obtained manually while conducting the essays. If however, video recording is used to film the behavioural tests, then the parameters could be extracted from the videotapes'.

As stated in step 2.8, the time spent by an animal swimming in any quadrant of the maze is simply the relative amount of seconds the animal is swimming inside that quadrant compared to the full 60s dedicated to the test.

-2.9.1: How are latency and velocity measured?

We added the following sentences to explain the meaning of each term (page 5, step 2.9):
Latency: time separating the start of the trial and the finding of the platform.
Velocity: Distance travelled while swimming per time unit.

-The timeline of training, probing, exposure, and testing is a little unclear. A diagram of the timeline would be helpful, for both acute and chronic exposure.

The overall timeline of the entire experimental protocol is now shown in a separate figure (Figure 1).

-Figure 1: Please include number of mice tested.

We added in both the text and the figure legend the number of animals used for each group.

•Discussion: What are the limitations of the protocol?

In the discussion, limitations of the protocol (such as potential CO2 intoxication) as well as ways to overcome these limitations are discussed. We also included a discussion of the possible adjustments of the protocol depending of the scientific question addressed.

• Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Indeed, all previous comments from the editor have been addressed in this new version as well.

• Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

We read the final draft at multiple occasions to correct any remaining typos and errors.

• If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

We included a phrase “Re-print with permission from (reference#)” in the legend of figure1. We also contacted the editor of Metab Brain Dis journal for a permission to re-publish our figure. As soon as we get the permission, we will forward it to you.

* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

When available, we added the DOIs of the references mentioned in the paper.

Response to Reviewers' comments:

Reviewer #1:

The presented protocol is relatively straightforward, based on methods generally used in the field and I have no concerns over the accuracy of the authors' observations. However, I have to make a number of important points:

1. The presented results have been already published in the cited Metab Brain Dis 2014 paper. Panels A and B from the manuscript were published as Figure 1 graphs in the 2014 paper by Fifel, Bennis and Ba-M'hamed, while panel C graphically represents the data contained in text of results in the 2014 paper. Is there a re-publishing issue? In addition, having in mind the authorships and acknowledgments in these two manuscripts, it would be of interest to check and make clear in which of two mentioned laboratories the presented experiments have been performed.

Indeed the figures are reprinted from my previous study upon which this protocol paper is based. For this, we have asked for a re-print permission from the Metab. Brain Dis. Journal.
The work described in the current protocol paper was conducted in Caddi Ayyad. We have made this more explicit in the disclosure section of the draft in page 2.

2. There is no expanded description of the protocol used in the Metab Brain Dis 2014 paper. For example, what was the color of the pool and platform? What about details of lighting conditions? The details of housing conditions throughout the experiment are also necessary for any replication efforts.

We added as much details as possible regarding the color of the pool and lighting conditions to make replication of the protocol straightforward.

3. It is stated: "Give the mouse 60 s to find the hidden platform." Panel B demonstrates means of Day 1 escape latencies of some 65 s. This is a striking and serious discordance between the method description and (already published) results.

Indeed, the timing allowed for the animals to find the hidden platform is 60s per trial. However, as stated in the protocol, if the animals do not find the platform within those 60s, they are gently guided towards it. The few seconds that this additional manoeuvre takes were behind the latencies of ≈ 65s that we recorded in Day1 of our test. There is therefore no discordance between the description of our protocol and the results we obtained (and published).

4. As soon as on Day 3, control escape latencies reached 20 s or so, and on Day 4, no more than 10 s. The authors stated that they used a "large circular pool of about 90 cm in diameter and 35 cm high". The results suggest quite oppositely: the pool was not large enough. The acquisition task encountered a clear-cut ceiling effect. Most probably, the diameter of the pool adequate for the given setup would be 120 cm for mice (and 200 cm for rats).

We do not think that the diameter of the pool used in our study (90cm) is necessary associated with a ceiling effect. The latencies of <10s observed in Day4&5 are mainly due to training. Similar latencies have been observed in several comparable paradigms even with pools larger than 1m in diameter (Morris RG et al., *Nature*, 1982). We do however agree with the reviewer that pools with

a diameter of 120cm can also be used and lead to optimal outcomes. We added a sentence and a reference discussing the impact of pool's diameter of the performances of mice in the MWM in page 4 of our draft.

Reviewer #2:

Manuscript Summary:

The title and abstract correspond to the purpose and the contents of this manuscript.

The test MWM more than 30 years used in many different neuroscience applications. Since MWM has many modified versions. Authors propose modified step for animals inhalation. This is 5 min interval between 15 min inhalation. The main argument for adequate performance of MWM is possible accumulation of carbon dioxide (CO2) over time in the inhalation chamber.

But it could be Plexiglas box more than (L:30cm, W:17,5cm, H:15cm) volume.

We do agree with the reviewer that Boxes larger than the one we used could be used to expose the animals to the paint thinner. However, unless continuous ventilation is allowed through the boxes, we do not think that larger boxes will avoid potential accumulation of respiratory CO2. For this reason, we highly recommend the 5min interval between the two 15min inhalation periods if one is using the protocol of inhalation we describe in this paper.

Any way proposed protocol is correct as well observed results.

Every sreps of protocol clearly explained.

Some questions are rising on become familiar with manuscript.

Authors should note

- the conditions for control animals group. Control mice should keep into chamber the same time without thinner inhalation;

We would like to thank the reviewer for this reminder. Indeed, control mice are exposed to the same protocol except that thinner is not injected into the inhalation boxe. We added this just after step 1.4 of the protocol (page 3).

- the chemicals contain of "paint thinner" that used in this issue; it could be different chemicals with distinct effects.

Although Toluene is by far the main constituent of paint thinners, we agree with the reviewer that the exact composition of the thinner may differ depending on the manufacturer. Consequently the behavioural and pathophysiological effects following its inhalation may differ.

- argue that 5 days treatment enough for the evidence of cognitive impairment.

As we made clear in many occasions throughout the draft, 5 days of training in the MWM are largely enough to sort out any potential cognitive impairment following intoxication by inhalants.

Sincerely,

The authors