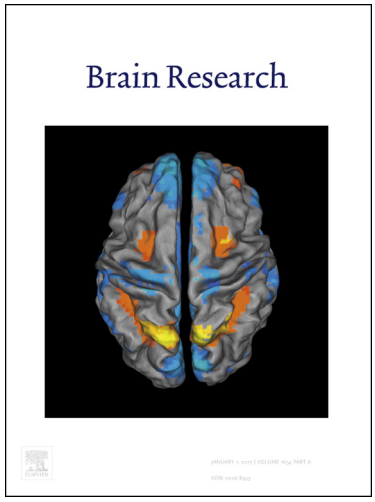
Offline consolidation of spatial memory: Do the cerebellar output circuits play a role? A study utilizing a Morris water maze protocol in male Wistar rats



Research report

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TITLE PAGE

Offline consolidation of spatial memory: do the cerebellar output circuits play a role? A study utilizing a Morris water maze protocol in male Wistar rats.

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Authors' contribution

P.A. conceived the study; P.A.,D.D.S.,F.F. designed the experimental protocol; P.A.,D.D.S.,P.F.,M.M.,F.F. carried out the experiments; P.A.,D.D.S., F.F. analyzed the data; D.D.S.,M.Z.,F.F. performed the statistical analysis; P.A., F.F. wrote the manuscript.

The corresponding author confirms that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. He further confirms that the order of authors listed in the manuscript has been approved by all of us.

Declarations of interest: none.

Highlights

- Post-training shutdown of cerebellar nuclei impairs consolidation of spatial memory
- Consolidation is impaired by inactivation of medial but not lateral structures
- Medially inactivated rats show lack of quadrant preference in the consolidation test
- Medially inactivated rats do not achieve optimization of behavioral trajectories
- Refined spatial map development requires cerebellar integrity during consolidation

Abstract

To address whether the cerebellum takes part to spatial memory consolidation related to navigation, male Wistar rats were trained daily (4 days), in a Morris water maze to found a submerged escape platform by use of distal cues (place training test). Retention of the allocentric map was evaluated in the probe test (without platform), before the place test. Bilateral shutdown of deep cerebellar nuclei was carried by infusion of the GABA A agonist muscimol (0,25 µl at 1 µg/µl) immediately after each place test. Histology revealed a dorsal dentate nucleus (DDN) group, with muscimol diffusion confined to dentate nuclei, and a ventromedial/dentate nuclei (VMDN) group, with muscimol additionally involving fastigial, interpositus and vestibular nuclei. In the place test, Vehicle, DDN and VMDN groups reduced latency and distance to the platform over the 4 days and within the single day, indicative of efficient acquisition and working memory; navigational trajectories however differed in that, while Vehicle and DDN groups evolved to use direct paths, VMDN group indulged to navigate in proximity to the platform, suggesting an impairment in refining the spatial map. In the probe test VMDN, unlike Vehicle and DDN animals, failed to develop a preference for the quadrant where the platform was previously located, indicating a consolidation deficit.

In conclusion, ventromedial cerebellar related structures may contribute to the process of consolidation of an allocentric spatial memory: their inactivation may have impaired the offline integration of idiothetic information with allothetic signals within the navigational network, leading to a coarse resolution map.

Keywords: Spatial navigation, Memory consolidation, Cerebellum, Behavioral strategies, Muscimol, Morris water maze.

1. Introduction

Navigation is the ability of animals to orient themselves in the environment in order to move toward a goal in the extrapersonal space (56). It requires the utilization of self-motion idiothetic signals and allothetic signals that provide information about the spatial relations between environmental cues. In a novel environment, during a phase of acquisition, both allothetic and idiothetic sensory information may be competitively or cooperatively utilized to gather knowledge of the surrounding environment, depending on the context in which navigation takes place and the reliability of the sensory information available (5). After acquisition, the significant spatial information undergoes a process of consolidation and storage in a long-term memory system so it can be retrieved when the animal is exposed again to the previous environment (18).

The various phases of acquisition, consolidation and recall of the spatial memory are associated with the activation of several neural structures, whose neurons process specific aspects of spatial information (19), for example place cells, head-direction cells, grid cells and border cells (8). The evidence of functional and anatomical segregation of neuronal structures handling principally allothetic or idiothetic spatial information led to propose the existence of parallel systems for organizing navigation behavior (11). Other studies, showing the presence in the hippocampus of neurons which maintain coherent couplings between self-motion (i.e., idiothetic) signals and environmental (i.e., allothetic) information propose instead that robust space coding requires a common network (see 5) of multiple brain regions (nodes) handling idiothetic and/or allothetic signals. Integration within the nodes may implement a unified space representation and may overcome the fact that neither idiothetic nor allothetic cues are by themselves immune from errors and limitations. Moreover, the single nodes may participate to the space representation differentially depending on the navigation strategy employed, the availability of sensory information and the phase of memory utilization (55).

The cerebellum was proposed to be part of the navigational network, providing the processing capabilities to manage complex idiothetic signals arising during navigation. The cerebellum is in fact involved in multisensory processing, sensorimotor transformations and motor control (37, 3); moreover anatomical and functional studies demonstrate several candidate bidirectional pathways of information transfer between the cerebellum and areas involved in navigation as the hippocampus, the frontal and the parietal cortices (see 50).

Rodents submitted to bilateral surgical ablation of the neocerebellum (lateral cortex including the dentate nucleus) or to stereotaxic lesions of the dentate nuclei (30) showed a slowing down in the acquisition of spatial representation of the environment, measured in the spatial version of the classical Morrison water maze (MWM) test (location of a hidden platform from random departure locations). Similar results were observed with stereotaxic bilateral lesions of the medial fastigial nucleus (31) indicating that the cerebellum may influence spatial memory acquisition acting via both medial and lateral output channels. Since in these experiments the navigation versus a visible cue (cued navigation) was not impaired, these results suggested that the deficit in acquisition was not purely of sensory-motor origin (30). Other evidences questioned this view: based on results in hemicerebelloctomized rats, it was suggested that the impairment to acquire the platform position was due to a

procedural deficit, i.e. to the inability to explore the pool with an efficient searching behavior while the mechanisms underlying the formation of the spatial map were unaltered (47).

Studies in the L7-PKCI mutant mice, a model with a deficit in cerebellar plasticity in absence of any motor-related deficit, confirmed a procedural deficit showing that trajectories were suboptimal due to cumulative motor execution errors over time (12) but also suggested that, besides providing the necessary procedural abilities, the cerebellum might also contribute to the construction of an integrated hippocampal spatial representations: in fact, in the dark and in the absence of the cues, when self-motion information must be predominantly used, the hippocampal place-cell system was unable to maintain stable place-cells fields, leading to impaired goal-directed navigation in the mutant mice (49).

Whereas the studies above indicate a role of the cerebellum in spatial memory related to navigation during the acquisition phase, the question is still open whether the cerebellum is also involved in the consolidation process by which fresh, initially labile, spatial memories are reorganized into stable memories (18). To address this issue, the present study investigated whether reversible bilateral shutdown of the cerebellar deep nuclei by locally administered muscimol [an agonist of the γ-amminobutyric acid type A (GABA-A) receptor] immediately after spatial training in the MWM impaired the development of memory consolidation. By utilizing a protocol promoting an allocentric spatial memory formation (i.e. guidance by spatial relationships of the distal visual cues located in the experimental room, 41) we could investigate the role of putative idiothetic signals processed by the cerebellum in the formation of a generalized stable space representation. Since spatial memory consolidation develops incrementally over short training sessions repeated daily (Place test), we repeated cerebellar nuclei inactivation after each of the four daily place test sessions. Given the time course of muscimol effects, with a rapid neuronal inactivation and a recovery within 24 hrs (36, 39, 2), the use of local muscimol injection shortly after each training session provides a method for influencing memory consolidation without affecting either acquisition or memory retrieval on the subsequent day. To avoid confounding effects due to the fact that for solving the task the animal must acquire some cerebellar-dependent procedural skills (such as climbing on the platform), all the animals were pre-trained on such aspects before being submitted to the spatial training protocol (33, 13).

In our study, we reasoned that inactivation to the deep cerebellar nuclei may be appropriate for the study of the role of the cerebellum in consolidation for various reasons. In particular, muscimol may have a direct effect on the plasticity of the nuclei by altering their inhibitory GABAergic input. Moreover, since these nuclei represent the only output structure of the cerebellar cortex, by depressing their activity at the infusion loci we may prevent the modulation of consolidation at target structures outside the cerebellum.

Potentially, the cerebellum may assist memory consolidation by elaborating idiothetic signals either within the archi-, spino- or neo-cerebellar circuits which through the deep cerebellar nuclei (fastigial, interpositus, dentate and also the vestibular nuclei) and their thalamic relays influence frontal and parietal forebrain areas (6). Moreover, the cerebellar output may target directly the hippocampus (see 50). In the present study, we decided *a priori* to inactivate the dentate nucleus whose axons target cortical areas particularly involved in spatial navigation: in particular in rats, dentate neurons give rise to a disynaptic cerebellar-thalamo-cortical projection

reaching the posterior parietal and the retrosplenial cortices (24, 50, 15). However, by histological identification of the injection cannula placement and drug diffusion, we were able to dissociate *a posteriori* the effects on consolidation produced by drug diffusion confined within the dentate nucleus itself from those observed in the cases in which the drug spread more ventro-medially thus targeting the more medial cerebellar nuclei (interpositus and fastigial) and the vestibular complex.

2. Results

2.1.1 Criteria for subdivision of the experimental population according to histological analysis.

In our experiments we localized the focus of the injection site on the basis of the reconstruction of the location of the tip of the cannula on post-mortem serial histological sections as illustrated in Fig. 2.

FIGURE 2

An approximate estimate of drug diffusion was obtained by evaluating the extent of the dye staining due to injection of a pontamine sky-blue solution, 1h before the animals were sacrificed and perfused.

From the combined analysis of the two parameters (location of the tip and diffusion of the pontamine sky-blue solution) it emerged that in the single animal, bilateral injections were almost symmetrically located while a higher degree of variability was observed between the animals in the mediolateral and ventrodorsal positioning of the cannula. In particular, in the muscimol treated animals, the tip of the cannula segregated in two discrete clusters, i.e. a ventromedial and a dorsolateral cluster. In the dorsolateral cluster, the pontamine sky blue dye stained the dentate nucleus and the lateral border of the interposed nuclei (mainly the anterior part), while in the ventromedial cluster, it additionally diffused to the anterior pole of the medial cerebellar nucleus and to the vestibular complex, including the lateral vestibular nucleus and nucleus Y. On this basis we split the muscimol treated animals into two different groups. Fig. 2 represents the position of the tip of the cannula reconstructed on frontal sections of the brainstem for the vehicle treated animals (Vehicle, n=7) and for the two muscimol treated groups, ventromedial/dentate nuclei (VMDN, n=4) and dorsal dentate nucleus (DDN, n=5). Histological analysis did not reveal damage to nuclear or cortical structures and the area damaged by the cannula was restricted to the cannula track.

2.1.2 Control for bias produced by acute effects of muscimol infusion.

When the animals were observed in the enlarged cage for 30 min, immediately after the end of the intracerebral infusion, none of them manifested gross motor impairment. A transient reduction in ambulation and rearing was observed in some animals, attributed *a-posterior*i in equal proportion to the two muscimol groups (i.e. VMDN and DDN groups). In particular in these animals, we did not observe signs of a left/right motor unbalance (head deflection, forced annular motion or roll and nystagmus) showing that our inactivation involved symmetrically bilateral structures (see 2.1.1).

2.2 Place training test on day 1: the pretreatment baseline.

In order to exclude that the *a posteriori* subdivision of the animal population based on histological analysis had introduced a bias with respect to different starting learning and visuomotor capabilities, a statistical analysis was carried out on data obtained in the first day of the Place training test (two blocks of trials), for the three experimental groups (Vehicle, DDN and VMDN). In our protocol, day 1 represents in fact the pretreatment baseline for all animals, since pharmacological treatments were given only at the end of the first Place training test

(Fig. 1). The GLM repeated measures *Group (3) x Block (2)* for latency and distance was applied, with the two blocks of trials as repeated measures.

The factor *Block,* but not the factor *Group,* was significant for both measures: latency ($F_{1,13}$=54.80, p<0.001, η^2p=0.808; 1-β = 1.000), and distance ($F_{1,13}$=60.45, p<0.001, η^2p=0.823; 1-β = 1.000). Latency and distance were reduced from the first to the second block of trials in all the three groups (Vehicle, DDN and VMDN): this suggests that the process of acquisition of the task is intact and starts already on the first training day. The lack of significance for the factor *Group* as well as for the interaction *Group x Block* in both latency and distance is an indication of the homogeneity of the animal population in the three groups.

2.3 Place training test over the four days: latency and distance.

On the whole testing period, i.e. along the eight blocks of the four training days, we registered in all animals a progressive reduction of latency and of distance covered to reach the platform. Fig. 3 represents data related to latency in the three animal groups. Besides the overall reduction of latency across days, a reduction of latency can be observed also within the single day between the first and the second block of trials, particularly on days 1 and 2, indicating the presence of an efficient working memory. Moreover, between two subsequent days, latencies in the first block were higher than in the second block of the previous day.

FIGURE 3

Data obtained in the subsequent four testing days were statistically analyzed in the three experimental groups (Vehicle, DDN and VMDN) with the GLM repeated measures *Group (3) x Block (8),* with the eight blocks of trials (two blocks/day) as repeated measures; a Greenhouse Geisser correction for violation of sphericity was applied. In the case of latency, there is an overall significant decrease, as shown by the significance of the factor *Block* ($F_{(3.47,\ 45.18)}$=25.85, p<0.001, η^2p=0.665; 1-β=1.000), in the comparisons *within* subjects. A significant effect of the factor *Block* was found also for distance ($F_{(3.23,\ 42.03)}$=26.13, p<0.001, η^2p=0.668; 1-β = 1.000) which gradually decreased for the adoption of more direct paths. The interaction between *Group x Block* for both latency ($F_{(6.95,\ 45.18)}$=1.31, p<0.267, η^2p=0.168; 1-β = 0.495) and distance ($F_{(6.45,\ 42.03)}$=1.23, p<0.308, η^2p=0.159; 1-β = 0.445) was not significant but the statistical result should be taken cautiously due to the suboptimal power. As a matter of fact, when Fig. 3 is visually inspected day by day, the VMDN group on day 2 and 3 began the first block of the day with higher latency values with respect to the other two groups; interestingly, the same VMDN group, in the second block of the same days reached similar levels of latency of the other two animal groups proving an efficient working memory.

2.4 Place training test over the four days: behavioral strategies.

In the training test, the analysis of the trajectories adopted by the animals to explore the maze and reach the escape platform may give indication on the stage of spatial map development and on the nature of information utilized. Fig 4 reports the % distributions of the five navigational categories (from lower to higher performance: *circling, extended searching, restricted searching, indirect finding and direct finding)* across the four training days

and for each group (Vehicle, DDN and VMDN)*: circling and extended searching* as well as *indirect finding and direct finding* were grouped together, also on the basis of their functional affinity.

FIGURE 4

The figure shows the tendency to a rapid path optimization with the abandon of the *circling* and *extended searching* (C+ES) occurring mainly in the transition from day 1 to day 2 in all the three animal groups. As for the *indirect and direct finding* (DF+IF) category, while control and DDN groups show a progressive strong increase along the four days, the VMDN animals remain at days 3 and 4 almost at the same level of the first two days. Finally, as for the intermediate *restricted searching* (RS) category, a reduction is observed from day 2 to day 4 in both Vehicle and DDN animals while VMDN group shows a more irregular pattern with high levels still at day 4 (Fig 4).

The percentage of occurrence for each category in the three groups was analyzed, after an arcsine transformation, with the GLM repeated measures *Group (3) x Day (4),* with the four days as repeated measures; a Greenhouse Geisser correction for violation of sphericity was applied. In the comparisons *within* subjects, the factor Day was significant for C+ES ($F_{2.30,\ 29.91}$=26.92, p<0.001, η^2p=0.674, 1-β = 1.000) and for DF+IF ($F_{2.76,\ 35.91}$=14.23, p<0.001, η^2p=0.523, 1-β = 1.000) but not for RS. In the comparisons *between* subjects, the factor Group was significant for DF+IF ($F_{2,13}$= 3.87, p=0.048, η^2p=0.373, 1-β = 0.593) and close to significance for RS ($F_{2,13}$=3.49, p=0.061, η^2p=0.350, 1-β = 0.547), i.e. the trajectory marking the beginning of an allocentric learning. Multiple comparisons by LSD showed a significant difference (p<0.05) between the group VMDN vs the group DDN both for RS (more represented in the VMDN animals), and for DF+IF (more represented in the DDN group).

2.5 Probe test: measures of retention along the four protocol days.

Starting from day 2 and for 4 consecutive days, a probe test, in absence of the platform, was applied at the beginning of each daily experimental session, in order to acquire specific indicators of consolidation of the spatial memory (Fig. 1). In the single trial of the probe test, we recorded the percentage of time spent in the quadrant where the platform was previously located i.e. the reference quadrant (% RQ). In addition, the following proximity parameters were measured: platform frequency, i.e. the number of visits to the area where the platform was previously located and target frequency, i.e. the number of visits to a circular expanded area centered on the platform. In Fig. 5, data of %RQ are reported: Vehicle and DDN groups show a gradual increase in time spent in the reference quadrant along the four testing days, indicating consolidation development, whereas the VMDN group split from the other two groups in correspondence of days 3 and 4, remaining on the same levels of the first two days. The VMDN group differed from the other two groups also for the two proximity parameters of platform frequency and target frequency, showing the same temporal pattern of %RQ.

FIGURE 5

The GLM for repeated measures *Group (3) x Day (4),* with Day as a repeated measure, was performed on the parameters of the Probe test; a Greenhouse Geisser correction for violation of sphericity was applied. In the case of RQ%, a significant difference was found for the factor Day ($F_{2.59,\ 33.76}$=10,62, p<0.001, η^2p=0.450; 1-β=0.994), as well as for the interaction Group x Day ($F_{5.19,\ 33.76}$=3.14, p=0.018, η^2p=0.326; 1-β=0.829), in the comparisons *within* subjects. The factor *Day* was significant also for Platform frequency ($F_{2.92,\ 37.91}$=5.10, p=0.005, η^2p=0.282; 1-β= 0.885) and Target frequency ($F_{2.54,\ 33.04}$=5.50, p=0.005, η^2p=0.297; 1-β=0.876). Marginal significances were recorded for the interactions *Group x Day* in Platform frequency ($F_{5.83,\ 37.91}$=2.23; p=0.062, η^2p=0.256, 1-β = 0.704) and Target frequency ($F_{5.08,\ 33.04}$=2.19, p=0.078, η^2p=0.252; 1-β = 0.645).

On the whole, the interaction effects underline a different temporal pattern in the three groups. Based on the significant interaction for the % RQ, the post-hoc analysis (LSD, p<0.05) indicates that in both Vehicle and DDN groups, day 1 values significantly differ from days 2, 3 and 4, due to an increment along days; on the contrary, in the VMDN group, no differences are found, since the percentage of time spent in the reference quadrant remains stable along the four days i.e. around 25%, a value indicative of a casual distribution.

2.6 Cue test: control for sensory motor deficits.

On day 5, just after the last probe test, a Cue test, in presence of a visible platform, was applied, in order to evaluate a possible impairment in sensory motor abilities. In this test, due to the visibility of the target the animal utilizes a taxis strategy not based on map construction; this strategy requires an active explorative drive, together with complex motor abilities such as the transformation of multimodal sensory information into a goal directed motor behavior.

The GLM repeated measures *Group (3) x Block (2)* for latency and distance was applied, with the two blocks of trials (four trials/block) as repeated measures. In the comparisons *within* subjects, the factor *Block* was significant for both latency ($F_{1,13}$=27.75, p<0.001, η^2p=0.681; 1-β = 0.998) and distance ($F_{1,13}$=42.69, p<0.001, η^2p=0.767; 1-β=1.000), but in the comparisons *between* subjects no *Group* effect was found: both latency and distance were therefore reduced from the first to the second block equally in all the three groups.

The ability of the three animal groups (Vehicle, DDN and VMDN) to carry out the test successfully suggests that the ability to utilize proximal cues is intact and rules out the presence of pure sensorimotor deficits, which could have been caused by damage of the neuronal structures following repetitive injections. Of particular interest is the absence of performance differences in the cue test between muscimol treated animals and controls, indicating that muscimol did not impact on integrative visuomotor behavior 24 h after the injection.

3. Discussion

This study was designed to assess the role of the cerebellum on the consolidation of the spatial memory related to navigation. In particular, we investigated whether reversible shutdown of the cerebellar deep nuclei by locally administered muscimol (a GABA-A agonist), immediately after spatial training in the Morris water maze (MWM), impaired the development of memory consolidation.

Histological analysis revealed that the location of the injection sites was symmetrical on the frontal sections; on the other hand the lack of signs of laterality in post-injection behavioral observations represents evidence of symmetrical inactivation of the neuronal structures. In the muscimol treated animals the locations of the injection site was distributed in two clusters: in one, injection remained confined within the lateral structures; in the other, the drug reached medial deep cerebellar structures as well. We therefore had to split the original muscimol treated animals into two groups which differed for the mediolateral extent of cerebellar nuclei shutdown. These two treatment groups differed in several measures of the behavioral tests applied; with a prudential attitude due to the low sample size, these results suggest a substantial and not occasional difference between lateral and medial structures on spatial memory consolidation.

3.1 Effects of post training cerebellar shutdown on the performance of the probe test.

In order to assess the role of the cerebellar nuclei in spatial memory consolidation we relied primarily on data obtained from the 60 sec probe test given 24 hours after each acquisition test. Consolidation can be in fact satisfactorily measured only after an adequate delay after training in the place test; this delay also minimizes the role of working memory in contributing to retention. Baldi et al. (7), compared the results of a probe test applied daily before the place test (i.e. 24 h after the previous training session) with the results obtained in a probe test applied immediately after the place test: they showed that the behaviour in the 24 h delayed probe test, similar to our protocol, reflects consolidated spatial information corresponding to long-lasting reference memory.

In the comparisons between groups (Vehicle, DDN, VMDN), differences in the degree of consolidation parameters emerged between the groups treated with muscimol (VMDN, DDN). The animals in which inactivation was restricted to the dentate nucleus (DDN) did not differ from controls, both increasing the time spent in the reference quadrant up to around 40% at day 4, indicative of consolidation. The latency in developing a clear quadrant preference attained at days 3 and 4 is in line with data in literature (7) and shows that our behavioral protocol was able to induce a consolidated spatial map. In contrast, the animal group in which inactivation additionally involved the more medial (fastigial and interpositus) cerebellar nuclei and the vestibular nuclei (VMDN) failed to show reliable signs of consolidation: at days 3 and 4, no quadrant preference developed as indicated by the fact that the time spent on the reference quadrant remained on the same levels of the first two days, i.e. around 25%, a value indicative of a casual distribution.

3.2 Effects of post training cerebellar shutdown on the performance within and across days in the place training test.

If we focus on the single day test, our data indicate that the deficit in the development of consolidation in the VMDN group does not depend upon an impairment in the acquisition of information within the daily spatial

training. In each of the 4 days of the place training test, a shortening of the escape latency between the first block (trials 1–4) and the second block (trials 5–8) was observed in all the three groups (Vehicle, DDN, VMDN); the decrease in latency was particularly evident during the first days of training. This finding suggests the efficiency of the working memory in utilizing the information experienced during the first block and/or in recalling the previous experience after reexposure to the task.

If we observe the evolution across the entire period (4 days) of the place test parameters, the performance was characterized by a significant progressive shortening of mean escape latency and travelled distance, two highly correlated measures (57); all the three animal groups reached comparable levels of performance at the end of the training sessions (place 4). The evolution across days depends in fact on the previous, day by day exposure to the task. Although a significant difference between groups was not revealed by the statistical analysis, likely due to the small sampling size, visual observation of latency values of the three groups suggest that some differences exist in an earlier phase. In particular, on day 2 and day 3, higher starting latencies were visually observed in the first block of the place test in the VMDN animals, which may be interpreted as indications of impairment in retention of the previous experience in the maze.

3.3 Analysis of the navigational strategies in the place training test may shed some light on the nature of the consolidation deficit.

As outlined in the previous section, we observed a shortening of latency and distance across days in the place training test affecting, albeit with some differences, all the three animal groups. In the MWM, the stressful condition associated to the test motivates an animal to reduce the time and distance to reach the escape platform (41); however, this goal may be reached either by optimization of non spatial searching strategies or by utilizing a newly developed allocentric spatial map of the environment. We utilized the analysis of the evolution across days of the navigational strategies to detect changes in the nature of the information (egocentric vs allocentric) the animal is using during maze exploration and to mark the beginning of allocentric learning in the place training test. It is known that in unimpaired conditions, learning the task occurs through progressive steps, with the transition from initial movement along the edge of the tank (circling, C), to a random exploration of the pool (non spatial searching strategy, i.e. extended searching, ES), followed by a focused exploration in the vicinity of the platform (restricted searching, RS); the latter behavior marks the transition from an egocentric to an allocentric spatial strategy and the beginning of the allocentric map construction (41,51). As the cognitive map is further strengthened and refined, this focused searching strategy narrows in precision to direct swims toward the platform (DF+IF: direct and indirect finding).

Analysis of the behavioral strategies revealed that independently of the group there was a rapid tendency to the abandon of the less efficient strategies, i.e. circling and extended searching; this effect, starting already on day 1 (i.e. before pharmacologic treatment) and progressing along days was likely due to the preliminary experience in the non spatial pretest (33, 13), which may have accelerated the first step of learning mainly related to procedural aspects. Most importantly, a group effect in the statistical analysis was found, which showed that RS was more utilized by VMDN animals with respect to DDN animals while the opposite was found for IF+DF; on the all,

whereas Vehicle and DDN rats progress to use almost exclusively the more direct paths (direct and indirect finding), the VMDN animals persist across days on the intermediate restricted searching, i.e. continue to navigate in proximity of the platform location without directly reaching it, indicating an impairment in the optimization of the spatial map .

3.4 Converging evidence of impaired consolidation in the VMDN group derived from the probe test and the place training findings.

As discussed in 3.1, a deficit of spatial memory consolidation affected the group of animals in which the drug diffused to medial structures, i.e. the VMDN group: despite the low number of animals, this conclusion is grounded on the statistical analysis of data in the probe test which showed sufficient power and on the congruence between intra test measures.

When comparing the performance in the probe test with the acquisition place test at the end stage of the protocol, the VMDN animals failed to show a quadrant preference in the last probe test (at day 5), when in the preceding place training test (at day 4) all the three animal groups had reached comparable performance, in terms of latency and distance. Therefore, a difference in the degree of performance in the acquisition task cannot explain the differences on the probe test behavior. Along the whole acquisition test however, differences in the behavior of the three animal groups were observed, which in the light of the probe results, can be interpreted as hints of impaired consolidation. As underlined in 3.2, at an early phase in the training protocol, i.e. on day 2 and day 3, higher starting latencies were visually observed in the first block of the place test in the VMDN animals, which may be interpreted as indications of impairment in retention of the previous experience in the maze. In a later phase, as underlined in 3.3, the statistical analysis of the navigational strategies, adopted in the place test by the VMDN animals, shows a lack of progression to direct finding and the persistence in the use of the restricted strategy. This effect could be interpreted as incomplete/impaired ability, due to the cerebellar shutdown, to incorporate during the consolidation period the sensorimotor information essential to refine the spatial details of an allocentric map leading to a map with a low spatial resolution (see 3.7).

3.5 Cerebellar shutdown does not impair the visuomotor abilities in the maze after 24 h.

Results obtained in the cued place test performed on the last day and 24 h after the last muscimol injection, indicate a good performance in the visuo-motor task in all three groups. In this test, the introduction of proximal stimuli (the visible platform) leads the animal to employ preferentially a target approaching strategy, which requires the capacity to use the visual information to reach the goal. This result suggests the absence of pure motor as well as sensorimotor transformation deficits in all the three groups up to the end of the experimental protocol. Moreover, it suggests that the shutdown of cerebellar nuclei does not interfere with this type of navigation.

3.6 Cerebellar circuits implicated in the consolidation of the spatial memory related to navigation.

Cerebellar circuits are composed of segregated modules that form loops (52) establishing bidirectional connectivity between specific cerebellar nuclei and their cortical and subcortical targets. In our experiment,

consolidation was impaired only when nuclear inactivation targeted the medial loops (fastigial, interpositus and vestibular nuclei) besides the lateral loops, as in the VMDN group.

The absence of consolidation deficits when nuclear inactivation was restricted to the dentate nucleus is not easily explainable: dentate nucleus is a node in the loops connecting the neocerebellum to the neocortex and even in rats a cerebellar thalamic cortical projection to associative cortices as the posterior parietal cortex has been described (24). Parietal cortex is connected to the hippocampus via indirect entorhinal cortex projections and is critical for spatial orientation in the rat (19, 11). If we postulate that the information essential for consolidation is conveyed by utilizing both the lateral (DDN) and medial (VMDN) cerebellar loops, the simultaneous block of the two loops may have impaired the capability of the navigational network to compensate for a local transient damage. A more simple explanation can however be proposed, i.e. that, in a functional segregation design, the medial structures have a more prominent role in spatial memory related to navigation since they are particularly engaged in receiving information from self motion cues (see 3.7).

Indeed, not only lateral but also medial output channels may target, through a thalamic relay (50, 53, 29), a variety of other areas involved in navigation including parietal, prefrontal, limbic and insular cortices (24, 28, 25). Medial cerebellar structures may also have a direct or indirect (via the ventrolateral nucleus of the thalamus) projection to the hippocampus (27, 43). Additionally, the medial cerebellum, both the anterior and posterior vermis, may affect the navigational network through its strict anatomic and functional links with the vestibular complex which processes self motion information. The zone B of the anterior vermis controls the lateral vestibular nucleus (Deiters nucleus) which projects to the fastigial nucleus while nodulus, uvula and flocculus target the superior, medial and descending vestibular nuclei as well as the nucleus prepositus hypoglossi and nucleus Y (29, 53, 60). Ascending pathways from the vestibular nuclear complex have been described which in turn may affect the navigation network (29, 53, 60): 1) an indirect pathway to the hippocampus (via nucleus reticularis pontis oralis, supramammillary nucleus and medial septum), which may affect the place cell system; 2) a pathway to para and post parasubiculum/medial entorhinal cortex (via supragenual nucleus, dorsal tegmental nucleus, lateral mammilary nucleus, anterodorsal thalamus), which may feed the grid cells and border cell system; 3) a projection to neo-cortical areas including the parietal cortex (via thalamic relays) for the perception of body motion and spatial orientation.

3.7 Nature of the information processed by the cerebellum during the consolidation phase.

As suggested in 3.6, the medial cerebellar structures may affect spatial memory since they receive and integrate information from self motion cues. For example, idiothetic information coming from vestibular otolith organ which senses linear acceleration (translation and tilt) and from the semicircular canals which sense rotation (angular velocity in 3D) is integrated at the level of fastigial, interpositus and vestibular neurons (26). Moreover, these nuclei and the overlying cortex also process stimuli from neck and limb proprioceptors regarding the position and movement of the body segments (20, 14, 34, 10). It is believed that cerebellum through the appropriate coupling of these multimodal sensory signals contribute to the build of a unified egocentric representation of the body movement in space, solving also the ambiguities associated to the single sensorial modality (37, 38, 58, 32, 9).

Finally, by comparing the actual sensory signals with their prediction computed using the efferent copy of the motor command (40), the cerebellum can distinguish unexpected self-motion resulting from external factors and self-motion generated by voluntary actions (17). These cerebellar operations may be functional to navigation (49). Since our protocol required the learning of an allothetic space representation in order to solve the test requirements, the question arises why blocking the system processing idiothetic information, specifically the medial nuclei, compromises the development of an allothetic-based spatial learning. According to Arleo and Rondi-Reig (5), idiothetic information might provide the spatial framework in which allothetic local views might be tied as the exploration of a novel environment proceeds. In an elegant computational model, Passot et al. (45) designed two cell populations driven by allothetic (vision) and idiothetic (path integration) signals. The idiothetic network is fed by cerebellar modules which provide an estimate of the future state of the whole body using idiothetic information and an efferent copy of the motor command. Both networks converge onto a third downstream network of hippocampal place cells to generate a stable space representation consisting of localized place fields similar to those found in hippocampal CA3-CA1 regions. Moreover, turning off plasticity at cerebellar level (45) leads to disruption of the hippocampal spatial code and produces a deficit in navigation similar to that observed in L7-PKCI mice lacking cerebellar plasticity, i.e. impaired goal directed navigation in conditions in which self-motion information must be predominantly used (49).

On the basis of the above reported evidences, a more precise interpretation of our data may be put forward and investigated in further experiments: the pharmacologic post-training shutdown of the medial cerebellar nuclei, carrying idiothetic signals, may have caused an incapacity to provide, during the consolidation period, the appropriate spatial framework in which allothetic local views might be tied, a requisite for a robust map to develop and consolidate.

3.8 Mechanism of cerebellar-dependent consolidation and their temporal aspects.

The involvement of the cerebellum in the consolidation of other forms of memory has been studied in a complex model in humans (21) and in simple reflexes in non-primate animals (59, 54, 4, 22) but, at our best knowledge, not in spatial memory tasks as in the Morris water maze.

In general, it is suggested that the formation of long-term memory is paralleled by the development of a series of plastic events at neuronal and/or at the network level. Therefore by blocking the cerebellar nuclear activity, we may have interfered with plasticity at the local cerebellar circuitry or at downstream circuits leading to a global network reorganization.

As for the local mechanism, given the convergence on the nuclei (including Deiters) of excitatory mossy fibers collaterals and inhibitory GABAergic projections from Purkinje cells, muscimol may have disturbed the GABAergic modulatory process influencing plastic changes in the deep nuclei (23).

The block of the nuclear output may have also blocked the rewiring of the entire navigational network, i.e. impaired plastic changes outside the cerebellum. According to the active consolidation hypothesis, reorganization occurs by a process of off-line replay of firing patterns assembled during the active behavior; reactivation takes place during rest immobility periods or during post-training slow-wave sleep (SWS, 44) or REM sleep (48) and

initiates waves of synaptic consolidation that gradually support changes in the morphological neuronal network structure. The cerebellum may be part of the reactivated network and be the target or the promoter of a global network reorganization given the evidence that a dialogue between the cerebellum and the forebrain may occur in the different animal states (waking and sleep) favouring the consolidation of waking experiences (1). It is in these phases of reactivation that idiothetic and allothetic signals may interact.

Plasticity events leading to consolidation may occur at different time intervals with respect to the learning experience. In our case, given the short time course of muscimol, injected each day just after the last place training trial, we may have blocked early consolidation processes. The drug may have interfered with the consolidation process during the waking phase since our rats, acclimatized to a light-dark inverted cycle, spent most of the time in a waking state in the period of the muscimol action. We cannot exclude however that we may also have interfered with consolidation occurring during sleep, for examples during the brief naps of the rats.

4. Materials and methods

4.1 Animals. Experiments were performed on 16 adult male Wistar rats (Charles River, Como, Italy), weighting 250-350 g (about 8 weeks of age). Rats were individually housed and kept under standardized conditions with food and water *ad libitum* on an inverted light-dark schedule (light: dark 12:12, light on at 8.00 pm, light intensity 100 lux, T=22-24 °C). Rats were allowed 7 days to acclimatize to their housing environment before being submitted to surgery.

Procedures were approved by the animal review board of the local committee on use and care of animals at the University of Siena (CEL.AOUS.29.03.2010). All efforts were made to minimize the number of animals used and their suffering in accordance with the European Community laws and NIH guidelines on animal care.

4.2 Surgical procedures. Under pentobarbital anaesthesia (70 mg/kg i.p.) and aseptic surgical conditions, rats were stereotaxically equipped with two chronic guide cannulae (24 G, OD 0.56 mm ,Plastic One Inc., 6591 Merriman Rd, Roanoke, VA 24018), symmetrically positioned at the following coordinates: Bregma AP -11.30 mm; ML 3.4 mm;l DV -5.0 mm, according to the atlas of Paxinos and Watson (46). The cannulae were secured to the skull with dental cement and screws. A solid cylindrical dummy cannula (OD 0.30 mm, Plastic One Inc.) was then inserted on the guide cannula to prevent its occlusion. Following surgery, all animals received an i.m. antibiotic injection (cefoperazone*;* Malesci, Florence, Italy*,* 250 mg/kg) and the wound was treated with a local anaesthetic (lidocaine cloridrate). Animals were allowed 9 additional days to recover before entering the behavioral training.

4.3 Water maze apparatus. The Morris water maze (MWM, 41) consisted of a circular pool of 180 cm diameter filled with water located in a room with several strongly contrasting extra maze cues. Four points, spaced at 90° around the circumference of the pool were arbitrarily designated North (N), South (S), East (E) and West (W) and, on this basis, the pool area was divided into 4 quadrants (NW, SW, NE and SE). When required by the test, a circular platform (12 cm diameter) was placed inside the pool in the middle of one of the quadrants approximately 45 cm from the side walls: the quadrant where the platform was located and the visibility of the platform varied according to the test, as described below. The water inside the pool (warmed at 25 ± 1 C°) was kept at the level of 12 cm below the border of the pool; it was made opaque by non toxic odorless tempera paint (Redmix, Lefranc & Bourgeous, France) to prevent visualization of the escape platform when placed below the water level. A videocamera (Sony AVC-DCSCE) hanging above the tank was used for video recording the behavior of the animals in the pool during the tests. The video was offline analyzed by using the software “The Observer XT” (Noldus Information Technology, The Netherlands) for quantification of the single parameters; in addition, the path followed by the animals was hand drawn on a paper by a blind observer and the distance travelled was later measured through an Electronic map measurer (K & R 154080).

4.4 Behavioral procedures. The protocol illustrated in Fig. 1 was designed to investigate whether consolidation of an allocentric spatial memory acquired in the Morris water maze was disrupted by transient pharmacological inactivation of the cerebellar nuclei performed just after each training phase.

FIGURE 1

Rats were randomly assigned to two treatments (muscimol or vehicle), as specified below. Blinding procedure required assignment of separate roles to the authors during the experimental sessions. A first group of experimenters assigned a progressive number to the animals and drew lots for the numbers in order to divide animals in two macro groups, indicated with capitol letters (A and B), then they sorted the order in which the animals entered in the daily maze protocol with the restriction that A and B groups should alternate; a second group of experimenters randomly allocated the two macro groups to the two treatments, muscimol and vehicle. Behavioral data were collected by the first experimenter group who was blind to the treatment allocation, and only referred to the macro groups A and B. The second group performed the intracerebral substances injections at the end of each daily session.

Animals were tested during the dark period of the cycle (i.e. between 9.00 am – 2.00 pm). They entered in the five day protocol in groups of 7-9 rats balanced at best for the ratio between muscimol and vehicle animals and in an alternating order for muscimol and vehicle animals; this order was maintained across testing days so that each animal was tested at the same time each day, allowing constant intervals between drug deliveries and behavioral testing and minimizing the influence of circadian factors (see Fig. 1). The entire daily procedure for each animal was carried out in a restricted time period (around 30 min). At the beginning of each behavioral test, the animal was removed from its home cage and placed in a warm box in the experimental room for a period of adaptation of 15 min.

A pretraining, non spatial test (42) was applied 9 days after surgery and 3 days before entering the behavioral protocol, in order to train the animals in the pool in the procedural aspects of the experimental setting. It consisted of a one day test (2 blocks of 4 trials; trial duration: 60 sec) performed in a room without visual cues and light sources with the exception of a red light source (invisible to rats). The platform was hidden 2 cm under the water level and moved pseudo randomly to a new quadrant after each trial with the same sequence across animals while the starting position of the animal was fixed (E, entry point). Due to lack of cues and the random position of the platform, rats did not acquire spatial cognition of the platform location relative to themselves or to the environment; however, they became aware of the existence of an escape platform and familiarized with the procedural skills such as navigating in the pool and climbing on the platform.

4.4.1 Place training test: applied daily for 4 subsequent days (days 1-4), to promote the acquisition of the location of an hidden platform in the presence of allocentric cues (see Fig. 1). It was organized in 8 trials each day (2 blocks of 4 trials: 3 min interblock interval, trial duration: 60 sec, intertrial interval: 60 sec). The rats were released into the water with their head pointing to the tank wall; in the interval between trials, they were returned to a warm box in the vicinity of the tank. In the apparatus, due to a dim white light source, extra maze cues were clearly visible: the platform was hidden under the water level (2 cm) and kept in the same quadrant (NE) during all trials, while the entry point of the animals (N, S, W, E) varied across trials (but remained constant across rats) according to a pseudo randomized sequence that ensured that two subsequent trials did not have the same entry point. Once animals reached the platform they were left on it for 60 sec. Animals that failed to find the hidden platform within 60 sec were gently guided towards it and left there for 60 sec. Since the starting point of the animal

changed at each trial the path needed by an animal to find the platform changed also at each trial; given that the platform remained fixed relative to the environment**, t**his version of the water maze test forced the animals to use distal instead of self-motion cues, i.e. to use preferentially an allocentric strategy. Immediately after the end of the daily training phase each animal received an intracerebral drug injection as outlined in paragraph 4.7.

4.4.2 Probe test: applied for 4 days, just before the place training test on days 2, 3 and 4, and on day 5, just before the Cue test (see Fig. 1). It consisted of a single navigation trial during which the platform was removed, while extra maze cues remained visible: the rat was placed in the pool at the N entry point and left swimming into the water pool searching for the removed platform for 60 sec. Probe test is considered a robust test to study spatial memory consolidation on the basis of the preference for the quadrant where the platform was located during the place test, and of other proximity measures (35).

4.4.3 Cue test: applied on day 5 just after the last probe test and 24 h after the last intracerebral pharmacologic treatment (see Fig. 1). In this test (2 blocks of 4 trials: 3 min interblock interval, trial duration: 60 sec, intertrial interval: 60 sec) the platform was visible, emerging 2 cm above the water surface, and was moved pseudo randomly in each of the 8 trials ensuring that two successive trials did not have the same platform position. In contrast, the starting position of the animals was fixed (entry point: N). The cue test served as a control test to exclude pure motor deficit as well as deficit of sensorimotor integration during navigation.

4.5 Behavioral Analysis. For each single trial of the various tests, quantification of performance was as follows:

4.5.1 Place training test: 1) *escape latency*: time to reach the platform, i.e. the interval, in sec, between the time of release of the animal in the pool and time of the climbing on the platform. If the animal failed to find the platform within the assigned time limit (60 sec), the trial latency was scored as 60 sec; 2) *distance*: path length, i.e. the total distance travelled by the animal, in cm (in a scale 1:10), during the time interval indicated by the latency. In addition, the path traced by the animal was used to define five navigational strategies, as follows: *circling* defined by peripheral swimming at the tank wall; *extended searching* defined as swimming in all pool quadrants, visiting the same areas more than once; *restricted searching* defined by swimming prevalently in the pool quadrant containing the submerged platform; *indirect finding* defined as reaching the platform through a direct trajectory with little path deviation and *direct finding* defined as direct straight pointing towards the platform without any further exploration.

4.5.2 Probe test: 1) *percent reference quadrant time* (% RQ), defined as the amount of time that the rat spent within the quadrant where the platform was located during the place training test and expressed as percentage of the duration of the test (60 sec). Since each quadrant represents the 25% of the total pool surface area, a value of RQ=25% suggests that the rat uniformly explored the whole pool area without any preference; 2) *platform frequency* measured the number of visits to a virtual area (12 cm diameter) where the platform was previously located; 3) *target frequency* referred to the number of visits to a virtual circular area bigger than the platform (27 cm diameter), centered on the previous platform location.

4.5.3 Cue test: 1) *escape latency* and 2) *distance,* as defined for the Place test.

4.6 Statistics. The measures taken in the Place, Probe and Cue tests from individual animals were averaged by pooling the results within each group of animals. When appropriate, single trial data, obtained from individual animals, were averaged across blocks to obtain a single value (mean of 4 values) and then total averaged values were obtained by pooling the results within the group of animals.

A generalized linear model (GLM) analysis with repeated measures for blocks (in the Place and Cue tests) and days (in the Probe test and in the Place test for behavioral strategies) was applied; the Greenhouse Geisser correction was used to correct for violation of sphericity when required, and the corrected degrees of freedom detailed in the Results section. Post hoc comparisons were performed by means of LSD test (threshold for the significance was: $\alpha = 0.050$). For all performed tests, the effect size was estimated and the partial Eta-Squared (η^2p) reported; Power (1-β) was also calculated and reported. Arcsine transformation for percentage data was used for behavioral strategies. All tests were performed using the software IBM SPSS 20 (IBM Chicago, IL) .

4.7 Functional inactivation and temporal aspects. For injections, animals were placed in dry cages and transported in a quiet room adjacent to the maze, where they were gently restrained to facilitate injection procedures. According to the experimental group, from day 1 to day 4 of the testing protocol (see Fig. 1), animals received, within 5-10 min after the last trial in the Place training test, a bilateral intracerebral injection either of muscimol hydrocloride (Sigma Aldrich) dissolved in a vehicle (artificial spinal cord fluid, ACSF) or of the vehicle alone. In particular 0.25 µl of muscimol at 1 µg/µl or of ACSF were injected, over a 120 sec period, via a 1 µl Hamilton syringe connected to an internal cannula (31 G; OD 0.36 mm, Plastic One Inc.) whose tip protruded 1 mm from the guide cannula allowing to reach the dorsal surface of the cerebellar nuclei. After the injection, the internal cannula was left in place for additional 120 sec to allow diffusion and to minimize backflow, and then replaced with the dummy cannula. At the end of the injection procedure the animals were placed in an enlarged cage and observed for 30 min for qualitative behavioral abnormalities. The short interval between the end of the acquisition test and the muscimol injection is functional to blocking the process of consolidation at an early stage. The effects of the drug are reversible and reasonably they should not have lasted so long to interfere with the subsequent acquisition phase (place test) or with the probe test measuring consolidation after 24 h.

4.8 Histology. On day 5 just after the Cue test, animals were deeply anesthetized by pentobarbital (70 mg/kg, i.p.); then bilateral intra cerebral injections were performed with an equal volume (0.25 µl) of ACSF stained with 5% pontamine sky blue (Gurr) with the same procedures described in 4.7. After 1 h (to allow dye diffusion), animals received an overdose of anesthetic and were perfused with PBS followed with 10% formaldehyde. The brains were then removed and stored in 10% formaldehyde. Cannulae placement as well as the extent of diffusion of the equal volume of pontamine sky blue solution were verified on frontal sections (20 µm) counterstained with neutral red.

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Authors' contribution

P.A. conceived the study; P.A.,D.D.S.,F.F. designed the experimental protocol; P.A.,D.D.S., P.F.,M.M.,F.F. carried out the experiments; P.A.,D.D.S.,M.Z., F.F. analyzed the data; D.D.S.,M.Z.,F.F. performed the statistical analysis; P.A.,F.F. wrote the manuscript.

Declaration of interests

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Captions

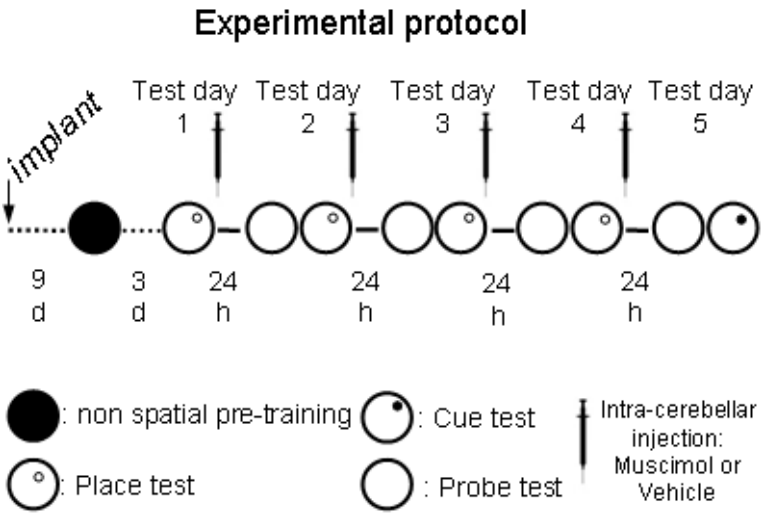
Fig.1. Timeline of the behavioral testing procedures in the Morris water maze. Protocol was organized over 5 days: a training test, Place test (submerged platform, 8 trials, 2 blocks of 4 trials, 60 sec/trial) was applied from day 1 to day 4. A retention test, Probe test (without platform, 1 trial, 60 sec) was applied on days 2-4 before the Place test and on day 5 before the visuomotor Cue test (visible platform, 8 trials, 2 blocks of 4 trials, 60 sec/trial). The injection symbol indicates bilateral intracerebellar injections given immediately after each Place test. Nine days (9 d) after the cannulae implant and 3 days (3 d) before the beginning of the behavioral protocol, animals were pretrained in a non spatial test.

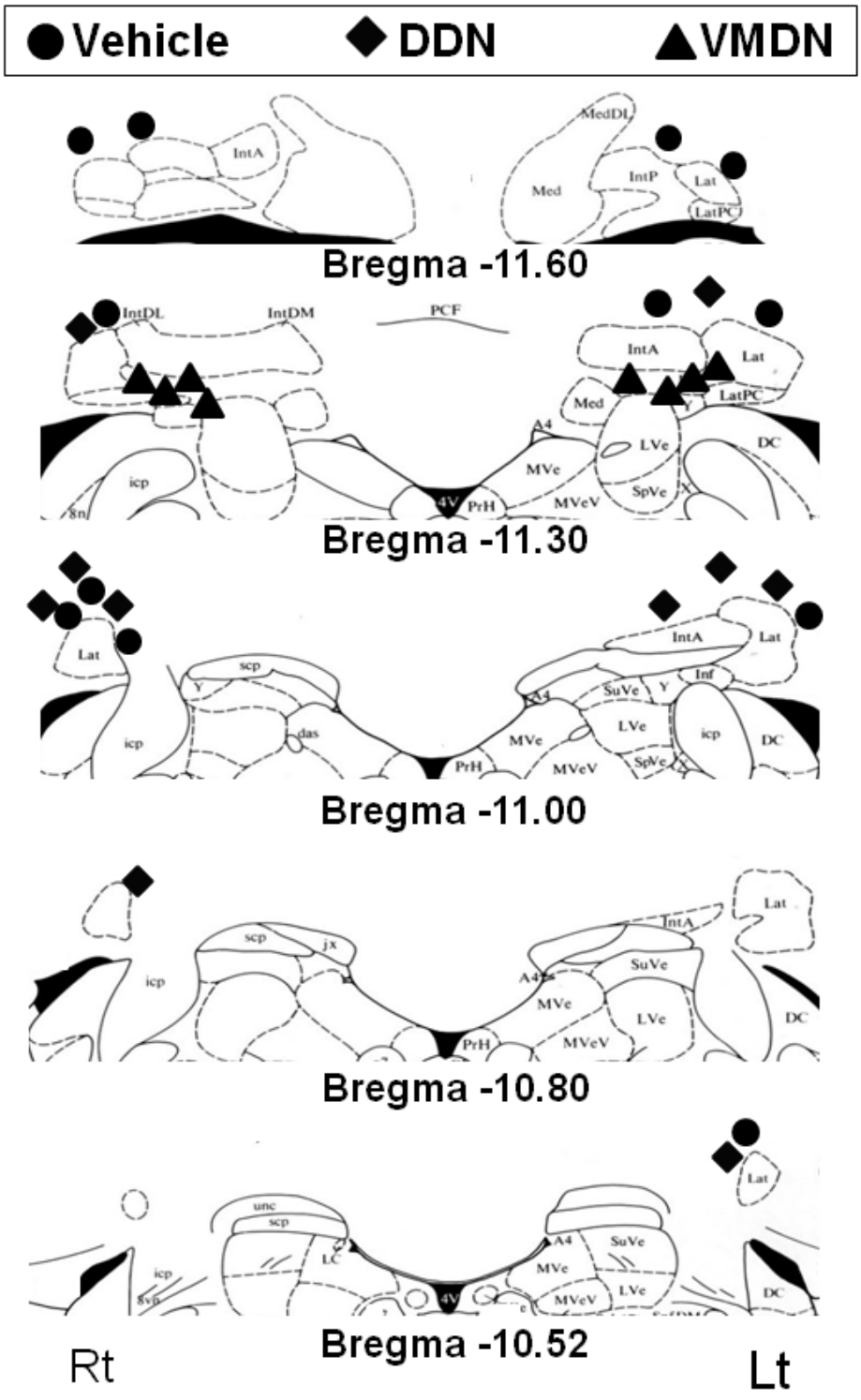
Fig.2. Location of the tips of the infusion cannulae. Camera lucida reconstructions of tip positions from serial sections (20 µm) of rat brainstem stained with neutral red. Tip positions are plotted on modified maps of coronal sections derived from the atlas of Paxinos and Watson (1998). Dots represent the sites of the tips for the animal group receiving vehicle. The tip sites of muscimol treated animals are partitioned in two groups, DDN, (diamonds), and VMDN (triangles) on the basis of the mediolateral/dorsoventral spatial gradient of the cannulae position. Note that in contrast to the Vehicle group, the spatial distribution of the tip position in the muscimol treated animals formed two clear segregated clusters. The two clusters also differed for the pattern of diffusion of the marker solution (pontamine sky blue at the same volume of the muscimol solution) to nearby structures.
Abbreviations: 8n/8vn, vestibularcochlear nerve; A4, adrenergic cell group; das, dorsal acustic stria; DC, dorsal cochlear nucleus; ICF, intercrural fissure; icp, restriform body; inf, infracerebellar nucleus; Int A, anterior interposed nucleus; IntDM , dorsomedial crest of the anterior interposed nucleus; IntDL, dorsolateral hump of the anterior interposed nucleus; Int P, posterior interposed nucleus; Lat, lateral cerebellar nucleus; jx, juxtarestiform body; LatPC, parvocellular part of the lateral cerebellar nucleus; LC, locus coeruleus; LVe, lateral vestibular nucleus; Med, medial cerebellar nucleus; Med DL, dorsolateral protuberance of the medial cerebellar nucleus; MVe, medial vestibular nucleus; MVeV, ventral division of the medial vestibular nucleus (magnocellular part); PCF, preculminate fissure; PrH, nucleus prepositus hypoglossi; scp, superior cerebellar peduncle; SpVe, spinal vestibular nucleus; SuVe, superior vestibular nucleus; Y, group Y of the vestibular nuclei; unc, uncinate fasciculus

Fig.3. Place test: **changes in escape latency across the four days protocol.** The plot reports the pattern of changes in escape latency to reach the platform (sec, in the y-axis) across the 8 blocks (2 blocks/day; 4 trials/block) of the 4 training days (in the x-axis) in the three experimental groups: Vehicle (n=7, dots), DDN (n=5, diamond) and VMDN (n=4, triangles). For each block, latency measures taken from single animals are averaged within each group (mean ± SD). Blocks 1 and 2 of day 1 represent the preinjection behavior. Note in all groups the progressive reduction of latency over the 4 days and within the single day indicative, respectively, of acquisition capabilities and of an efficient working memory. The VMDN group however, started the first block of day 2 and 3 with higher latency values.

Fig.4. Place test: changes in the utilization of the navigational strategies across the four training days. For the three experimental groups, Vehicle (n=7), DDN (n=5) and VMDN (n=4), histograms represent the frequencies of the searching strategies, expressed as a percentage of the strategies selected in the 8 trials of a given day (in y-axis): circling + extended searching (C+ES: top diagram), restricted searching (RS: middle diagram) and direct finding + indirect finding (DF+IF: bottom diagram). Within each experimental group the four bars report data from the four days of test with each bar reporting the grand average frequency of the group (mean ± SD). Note: in all animal groups the rapid abandon of C+ES; in the Vehicle and DDN groups the progressive increase across days of IF + DF strategies; in the VMDN group the persistence in RS strategy and the lower utilization of IF + DF.

Fig.5. Probe test: d**evelopment of the quadrant preference across the four testing days.** For the three experimental groups, Vehicle (n=7, dots), DDN (n=5, diamond) and VMDN (n= 4, triangles), the % time spent in the reference quadrant where the platform was previously located (RQ %, in y-axis) is plotted against the four daily tests (x-axis). Measures taken from single animals are averaged within each group (mean ± SD). Note that from day 3, Vehicle and DDN groups started to show a robust quadrant preference in contrast to VMDN group in which the RQ% remained around the 25%, indicative of a casual distribution.




Vehicle
DDN
VMDN
IntA
MedDL
Med
IntP
Lat
LatPC
Bregma -11.60
IntDL
IntDM
PCF
IntA
Lat
Med
LatPC
A4
LVe
DC
MVe
icp
4V
PrH
MVeV
SpVe
Bregma -11.30
Lat
scp
IntA
Inf
SuVe
LVe
MVe
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das
PrH
MVeV
SpVe
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Lat
IntA
scp
jx
icp
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Rt
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