

Neurobehavioral studies, in transgenic F3/CONTACTIN (C57BL/6J × CBA) mice, on cognitive and anxiety aspects during late-adolescent period

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Abstract. – OBJECTIVE: Besides than in the control of developmental events, axonal adhesive glycoproteins may be also involved in functions requiring fine organization and connectivity of the nervous tissue. We previously demonstrated morphological alterations and functional cerebellar deficits in transgenic mice (TAG/F3 mice) ectopically expressing the F3/Contactin axonal glycoprotein under the control of a selected regulatory region from the Transient Axonal Glycoprotein (TAG-1) gene. In the present study, the hippocampal function was explored by evaluating the ability of TAG/F3 mice to encode spatial and non-spatial relationships between discrete stimuli and to analyze an anxiety-related behavior.

MATERIALS AND METHODS: To the first end, mice were placed in an "open-Field" containing five objects and, after three sessions of habituation (S2-S4), their reactivity to objects displacement (S5-S4) and object substitution (S7-S6) was examined. To the second end, mice were placed in the "elevated zero maze", a standard test to explore the anxiety-related behavior, in order to study, in transgenic mice, the effects of F3 misexpression on emotional reactivity by measuring the avoidance of the unsheltered open sectors.

RESULTS: Statistical evaluations of reactivity to object novelty, TAG-F3 mice showed a lower DO exploration with respect to wild-type mice and, regarding DOs, TAG/F3 mice interacted less than wild-type mice, showing an impaired spatial change response. Furthermore, the number of HDIPS in transgenic TAG/F3 mice resulted significantly lower with respect to the controls (wild type).

CONCLUSIONS: These results indicate that the coordinated expression of axonal adhesive glycoproteins may be relevant for the functional maturation of the hippocampus.

Key Words:

TAG/F3 mice, Axonal adhesive glycoproteins, Anxiety-related behavior, Reactivity to object novelty, Novel object exploration test, Elevated zero maze test.

Introduction

In the development of the Nervous System, the cell interactions are mediated by various classes of neuronal surface glycoproteins (cell adhesion molecules) including the Immunoglobulin Superfamily¹⁻³, in which is enclosed the mouse axonal cell adhesion molecule F3/Contactin glycoprotein (F3)⁴⁻⁶. The structural organization of the F3 is derived from the cloning of its c-DNA and reveals that this glycoprotein is encoded by a single-copy gene, mapping to band F chromosome 15 in the mouse⁷ and to chromosome 12 in the human genome⁸.

Structurally, the axonal cell adhesion molecule F3 is composed of 1020 aminoacids with a hydrophobic segment at both ends and a glycosphosphatidylinositol (GPI)-anchor in common with other membrane proteins^{4,5}. Moreover, the F3 presents about 30% of similarity in the

aminoacidic sequence with the transmembrane glycoprotein L1⁹ and a 50% of similarity with the (GPI)-anchored Transient Axonal Glycoprotein (TAG-1)¹⁰.

Moreover, F3 is classified in a group of axonal cell adhesion glycoproteins presenting a different expression during the development of the nervous system¹¹ which includes, in addition, TAG-1 (CNTN-2) (12), BIG-1 (CNTN-3) (14), BIG-2 (CNTN-4)^{12,13}, NB-2 (CNTN-5)¹⁴ and NB-3 (CNTN-6)¹⁵.

Furthermore, F3 is also stored in various species of animals during the phylogenesis such as the *Helix Pomatia*¹⁶, *Drosophila*¹⁷, chick¹⁸, mouse⁴⁻⁶ and humans⁸.

The F3 is considered a brain specific glycoprotein and ultrastructural studies in the nervous tissue demonstrate that F3 is expressed in neuronal and glial elements; in the neurons, during the development, it is early distributed on the neuronal cell bodies and later this localization is lost with concomitant and prevailing expression in the axons⁵, to which it exercises inhibitory effects on axonal growth^{19,20}. F3 is also expressed in the glial elements, such as oligodendrocytes²¹; this last data is in accordance with the role played by F3 in the early stages of myelination and in the organization of nodal and paranodal regions; furthermore, recently study suggest that F3 is involved in the signaling pathway which promotes oligodendrocyte maturation²². F3 is also involved in the myelination and in nerve pulse conduction; in fact, it is expressed at level of axonal nodal, paranodal and juxtaparanodal regions²³⁻²⁸ and in the conduction of nerve impulse^{27,29,30}.

In fact, at the level or the node of Ranvier, F3 interact with the B1 subunit of the Na channels⁷. Moreover, at the paranode level, the complex F3-CASPR (Contactin associated protein) mediated the interaction between axons and myelinating cells, which implies the interaction with the glial Ig superfamily component Neurofascin 155 and Nogo-A glycoprotein^{31,32}.

Moreover, in human astrocytic gliomas cells is observed F3 which, in this tumor, is a possible factor involved in glioma disaggregation contributing to the infiltration of glioma cells in human brain³³.

In the development of the central nervous system, an important aspect is the contrasting action between F3 and transient axonal glycoprotein (TAG-1), F3 plays an inhibitory role in the axonal growth (neurite elongation and regeneration)

and in mechanisms of differentiation and myelination of glial cells (oligodendrocyte) while TAG-1 performs positive effects^{19,22,34,35,20}.

In the development of the telencephalon, F3/Contactin glycoprotein (at embryonic day 16th) is expressed in the proliferative region of the ventricular zone (VZ) and in postmitotic neurons, localized in the layers II-III of the cerebral cortex³⁶, which originate from neuronal precursors located in the upper ventricular zone³⁷; in the hippocampus F3 is expressed in the pyramidal neurons of the CA1-CA3 zones; in the pyramidal neurons, the F3 is primarily expressed in cell bodies; subsequently, the expression is polarized in the axonal extensions; moreover, the F3 is also expressed in the granule neurons of the dentate gyrus³⁶.

In the cerebellum, F3 is involved in the maturation, elongation, myelination of the axons and is also an important component in the cerebellar synapsis machinery^{38,39}, presents significant expression at postnatal day 8 and is involved in important changes of the cellular expressions during the neuronal differentiation; in fact, during the development of the cerebellum, F3 is observed in different neuron types, in the granules at the birth and in the first postnatal week and in the Purkinje neurons where its expression begins at postnatal day 3 and shows the maximum levels at postnatal day 8^{5,36}. A lower expression of F3 is permissive for neurogenesis during the expansion of the neuronal precursor pool and contributes for a normal sequence of the cell type specification during corticogenesis⁴⁰.

Materials and Methods

Animals and Breeding Procedures

All animal experimentation was performed in accordance with the EU directive 86/609 EEC, with the guidelines released by the Italian Ministry of Health (D.L. 116/92), with the UK "Animals Act 1986" and associated guidelines and with the "Guide for the Care and Use of Laboratory Animals" as adopted and promulgated by the National Institutes of Health. According to the above guidelines, all efforts were made to minimize the number of animals and their suffering.

The generation of the TAG/F3 transgenic line has been previously described²⁰. Animals were allowed free access to food and water, housed at constant room temperature (20-22°C) and exposed to a light cycle of 12h/day (08.00-20.00h).

Pairs of females were placed with a single male in the late afternoon and inspected, on next morning, for the presence of the vaginal plug (gestational day 0, GD0). The day of delivery was designated as postnatal day 0 (PND 0) and the litter size were recorded and reduced to six males, when possible. Pups were then weighed and marked with black ink on their backs for individual identification. On PND21, pups were weaned and separately housed in cages identical to the home cage.

“Novel Object Exploration Test” Apparatus

The apparatus consisted of a circular open-field, 60 cm in diameter with 20-cm-high walls made of black plastic material, with a white painted floor divided into sectors by black lines. The open-field was located inside a soundproof cubicle and surrounded by a visually uniform environment except for a conspicuous striped pattern, 20 cm wide and 10 cm high (alternating 1.5 cm-wide vertical white and black bars) attached to the wall of the field. A video-camera, placed above the arena, connected to a video tape-recorder and a monitor, recorded all test sessions for subsequent replay and analysis. Five different plastic objects were simultaneously present in the open field: (1) a chromium-plated parallelepiped ($7 \times 4 \times 4$ cm) with irregularly distributed holes on all the sides; (2) a transparent cylinder with a conic top (diameter of the section: 8 cm; height: 6 cm); (3) a small ladder made of grey plastic material (height: 16 cm, width: 5 cm; number of steps: 10), inserted on a cylindrical basement (height: 2 cm; diameter: 7 cm); (4) a black Plexiglas cylinder (height: 10 cm; diameter: 5 cm) with an edge (height: 2 cm) on the top and (5) a red and white spool (height: 12 cm; diameter of the top and the basement: 5 cm) with a small electric light bulb fixed on the top.

The initial arrangement was a circular plate with a central object (B) as schematized in Figure 1. A sixth object (F) was used to examine the reactivity to non-spatial change. It consisted of two grey iron regularly pierced squares (10×10 cm) forming a 90° angle. During session 1 (S1), the mouse was placed into the empty open field and the baseline level of activity was measured. Before session 2, the striped pattern and the five objects were placed as in Figure 1a. Habituation on object exploration was recorded during session 2-4. Before

session 5 (spatial novelty), the configuration was changed by moving two objects: object B replaced object D, which was itself displaced at the periphery of the apparatus so that the initial square arrangement was changed to a polygon-shaped arrangement. Before session 7 (object novelty), one of the familiar not displaced object (E) was replaced by a new object (F) at the same location.

The test, performed between 09:30 and 14:30 h, consisted of seven successive 7-min sessions with 2 min inter-trial intervals during which subjects were returned to their home cage. At the end of each test, all the objects and the arena were cleaned with 5% ethanol-water solution in order to eliminate any possible olfactory cues. Mice were tested in late adolescence (PND 45). Experimental groups were the following: wild-type mice $n=10$; TAG/F3 mice $n=9$.

Data Collection in the “Novel Object Exploration Test”

During all sessions, the frequency of the following responses were measured: *rearings* (standing on hind legs), *wall rearings* (standing on hind legs and placing forelimbs on the wall of the arena), *grooming* (wiping, licking, combing

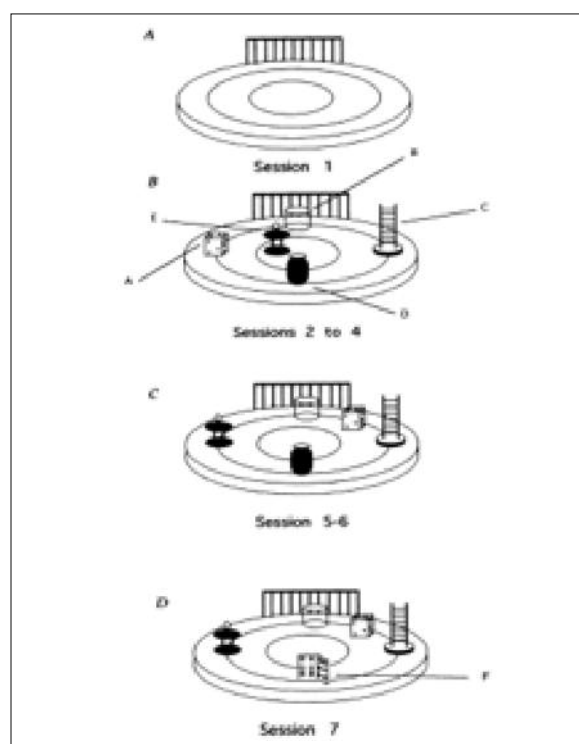


Figure 1. “Novel Object Exploration” Test.

or scratching any part of the body) and resting time. In addition, *locomotor activity* was assessed by counting the number of sectors crossed by the animal while moving in the open-field.

From S2 to S7, in addition to the above described behavioral responses, also the time spent by the animals in contact with the objects was recorded. Object contact is a behavioral response which has been used in previous studies in adult rodents in the same test and which seems to be sensitive to the changes occurring in the experimental environment^{41,42}. In order to measure object contacts which were both physical and visual, a contact was defined as the subject's snout actually touching an object. We assessed the habituation to the objects analyzing the duration and frequency of the contacts with the objects during S2-S4 in each group. Furthermore, we assessed the response to spatial change in S5, when the spatial arrangement of the objects was modified, comparing the two resulting variables referred to as DO (displaced objects) and NDO (non-displaced objects). DO corresponds to the mean time spent in contact with DOs B and D in S5 minus the mean time spent in contact with the same objects in S4, while NDO corresponds to the mean time spent in contact with NDOs in S5 minus the mean time spent in contact with the same objects in S4. Finally, in S7, the response to object novelty was assessed by comparing the two resulting variables referred to as NO (novel objects) and FO (familiar objects). NO corresponds to the time spent in contact with the NO 6 minus the time spent in contact with the object located in the corresponding position in S6, namely object 2. FO is the mean time spent in contact with familiar non-substituted objects minus the mean time spent in contact with these same objects in S6.

Statistical Analysis

One-way and two-way ANOVAs for repeated measures were performed to analyze data of novel object exploration test. Post hoc comparisons were performed using Tukey's test.

"Elevated Zero Maze Test"

According to the technique previously described by Cook et al 2001⁴⁴, the "Elevated Zero Maze" apparatus consisted in a black Perspex annular platform (40 cm diameter, 5 cm wide) elevated 108.9 cm above ground level and equally divided into four quadrants. Two opposite quadrants were enclosed by black Perspex walls (28.5

cm high) on both the inner and outer edges of the platform, while the remaining two opposite quadrants were surrounded only by a Perspex lip (1 cm high) which served as a tactile guide to animals on these open areas.

The apparatus was illuminated by a dim red light arranged in such a manner as to provide similar lux levels in open and closed quadrants (40-60 lux). A video camera, connected to video equipment in a separate observation room, was mounted overhead in order to record the behaviour of the mouse on the maze for subsequent analysis.

Procedure

Subjects were placed on a closed quadrant and a 5 min test period was video-tape recorded for subsequent analysis. The maze was cleaned with 5% ethanol/water solution and dried thoroughly between test sessions. Behavioural measures were as follows:

1. (**% TO**): Percent of time spent on the open quadrants expressed as the percentage of the total time of the test. Time on the open quadrants was timed from the moment in which all four paws of the rat were placed on an open section and ended when all four paws re-entered a closed quadrant.
2. (**HDIPS**): Number of exploratory head dips made over the edge of the platform, either from the exit of the closed quadrant or whilst on the open quadrant;
3. (**SAP**): Number of stretched-attend postures made from the exit of a closed quadrant towards an open quadrant. This exploratory posture could be described as a forward elongation of the body with static hind-quarters, followed by a retraction to the original position.

Testing was carried out in 90 day old mice between 13.00 and 17.00 hrs. Each group consisted of 10 animals.

Results

1. **Locomotor Activity.** The number of crossings performed by TAG/F3 mice during the "novel object exploration test" is not significantly different with respect to the controls both in males and females (Data not shown).
2. **Habituation of Object Exploration.** Object exploration scores during sessions 2, 3 and 4 (S2-S4) were analysed. A two-way ANOVA on

these data didn't show any significant effect on genotypes ($F_{1,17} = 1.16$; n.s., sessions ($F_{2,34} = 2.05$; n.s. and interactions ($F_{2,34} = 1.52$; n.s.). (Data not shown).

3. Pre-change response to object categories at S4. A two-way ANOVA on exploration scores regarding object categories subsequently displaced (DO) or not displaced (NDO) didn't show any significant effect on genotypes ($F_{1,17} = 1.60$; n.s.), object categories ($F_{1,17} = 3.52$, n.s.) and interactions ($F_{1,17} = 0.63$, n.s.) Data not shown

4. Response to Object Categories at S5. In S5, when the object were displaced, mice returned to explore (mean $\pm$ S.E.M.: wild-type DO = 18.45 ± 2.47 ; wild-type NDO = 11.53 ± 1.35 ; TAG/F3 DO = 5.44 ± 1.78 ; TAG/F3 NDO = 9.74 ± 4.30). Two-way ANOVA showed a significant effect on genotypes ($F_{1,17} = 6.47$; $p < 0.05$), no effect on object categories ($F_{1,17} = 0.31$, n.s.) and a significant effect on interactions ($F_{1,17} = 5.58$, $p < 0.05$). Post-hoc comparison (Tukey's test) showed a lower DO exploration in TAG-F3 mice with respect to wild-type mice ($p < 0.01$), (Figure 2A).

5. Response to Spatial Change (S5-S4). Time spent in contact with DOs and NDOs is shown in Figure 2B. A two-way ANOVA showed a significant main effect on genotypes ($F_{1,17} = 6.70$; $p < 0.05$), object categories ($F_{1,17} = 24.05$; $p < 0.005$) and interactions ($F_{1,17} = 14.40$; $p < 0.05$). Post-hoc comparisons (Tukey's test) revealed that in wild-type mice DOs were significantly more explored than NDOs ($p < 0.001$). Regarding DOs, TAG/F3 mice interacted less than wild-type mice ($p < 0.001$), showing an impaired spatial change response ($p < 0.001$) (Figure 2B).

6. Pre-change Response to Object Categories at S6. A two-way ANOVA on exploration scores regarding object categories subsequently substituted (SO) or not substituted (NSO), didn't show any effect on genotypes ($F_{1,17} = 0.11$; n.s.), object categories ($F_{1,17} = 3.69$, n.s.) and interactions ($F_{1,17} = 0.02$, n.s.), data not shown.

7. NO Responses (S7-S6). Two-way Anova on reactivity to object novelty did not show any significant effects on genotypes ($F_{1,17} = 1.33$; n.s.), object categories ($F_{1,17} = 3.52$; n.s.) or interaction ($F_{1,17} = 3.82$; n.s.). data not shown.

Elevated Zero Maze Test

The number of HDIPS in transgenic TAG/F3 mice resulted lower with respect to the controls. In particular, the HDIPS resulted statistically lower in male transgenic mice ($p < 0.001$) and female transgenic mice ($p < 0.025$) with respect to the controls (wild type) (Figure 3).

Discussion

In the development of the telencephalon, F3 (at embryonic day 16th) is expressed in the proliferative region of the ventricular zone (VZ) and in postmitotic neurons, localized in the layers II-III of the cerebral cortex³⁶, which originate from neuronal precursors located in the upper ventricular zone^{37,43}; in the hippocampus, F3 is weakly expressed until P3, after P8 is sharply expressed in the pyramidal neurons; in this neuron types of the CA1-CA3 zones it is primarily expressed in the cell bodies; subsequently, is polarized in the axonal extensions; moreover, the F3 is also ex-

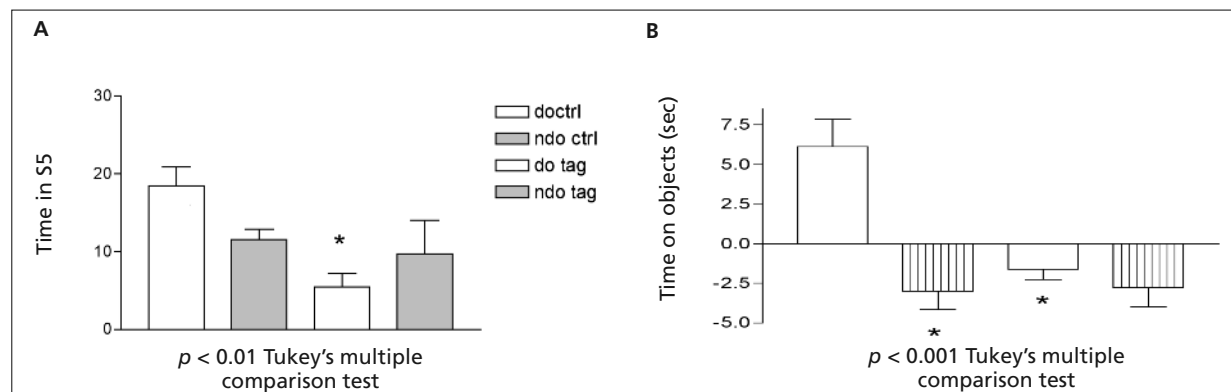


Figure 2. **A.** Response to object categories. **B.** Response to spatial change.

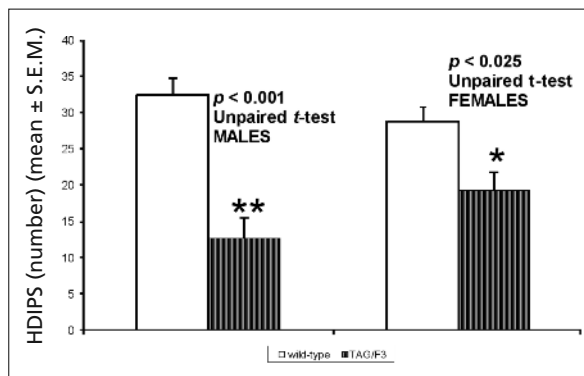


Figure 3. Number of HDIPS of TAG/F3 mice with respect to controls (wild type).

pressed in the granule neurons of the dentate gyrus³⁶. In the olfactory bulb at postnatal 0, F3 is highly expressed in the fiber layer in the axons of the olfactory neurons, and decreased through the internal granular layer; at postnatal day 3 a high expression of F3 is still localized in the olfactory nerve axons and nearby the region of the glomeruli; at postnatal day 11 the expression of F3 is localized in the superficial olfactory axons and in some mitral cells⁵. In the cerebellum, F3 is involved in the maturation, elongation, myelination of the axons and is also an important component in the cerebellar synapsis machinery^{38,39} and presents significant expression at postnatal day 8 and, is involved in important changes in cellular expressions during the neuronal differentiation, in fact, during the development of the cerebellum F3 is observed in different neuron types in the granules at the birth and in the first postnatal week, in the Purkinje neurons the expression begins at postnatal day 3 and the maximum levels are observed at postnatal day 8^{5,36}.

A valid experimental model for study the role of the F3 during the development of the central nervous system is a transgenic mice named TAG/F3, because in which the F3 expression is placed under the control of the TAG-1 gene regulatory sequences²⁰. Recently, studies suggest that the transgenic misexpression of F3 modified the cerebellar neurogenesis through suppressing precursor proliferation²⁰ and recently is reported that in the development of the cerebral cortex overexpression of F3 is involved in delayed corticogenesis; on the contrary, a lower expression of F3 is permissive for neurogenesis, during expansion of the neuronal precursor pool and contribute for a normal sequence of the cell type specification during corticogenesis⁴⁰.

Conclusions

This experiment was designed to examine the effect of F3/Contactin misexpression on habituation of object exploration and reactivity to spatial or to non-spatial change in TAG/F3 mice. The detailed ethological analysis of the behavior of the mice in a novel open field revealed time dependent changes in the frequency and duration of various behavioral patterns. Consistently with their natural behavioral response to a novel environment, control mice tended to display more fear-related behaviors such as thigmotaxis, exploration, stretch attend postures and returns in the first part of the open field and more of other locomotory and investigatory-associated behaviors such as walk and rear towards the end of the test. Other behaviors, such as grooming, tended to gradually increase with time. The location of the mice in the open field also showed a time dependent shift from the edges toward the center. This again is consistent with a time dependent reduction in anxiety and/or fear. Both rearing and grooming are responses related to habituation when exploring a novel environment. In particular, self-grooming is traditionally considered an adaptive response to novelty and to potentially stressful situations⁴⁴, which tends to increase with repeated exposure to a novel environment while locomotor activity tends to decrease. Recent data showing increase of grooming in an open-field test upon anxiolytic administration support the role of this behavior in counteracting novelty-induced stress⁴⁵.

In the presence of a cerebellar deficit, many postural and motor deficits are inescapably present. In spite of this, TAG/F3 mice succeeded in moving inside the arena and they habituated to the new environment as with the control animals. This observation is in agreement with data reported by Dahhaoui et al⁴⁶ in cerebellectomized rats and by Caston et al⁴⁷ in intact or cerebellectomized Lurcher mutant mice, suggesting that the cerebellum is not involved in the habituation of exploratory behavior. In session 5, wild-type mice showed a positive value of DO coupled with a negative value for NDO, whereas for TAG/F3 animals a positive value for DO was coupled with a positive value for NDO. These results suggest that control mice increased exploration selectively oriented towards displaced objects; on the contrary transgenic mice showed a general increase in exploration, not selectively oriented towards displaced objects.

However, the acquisition of a declarative competence of an environment does not require cerebellar involvement *per se*. Cerebellar mediation is fundamental in acquiring the procedural competence that appears to be a pre-requisite of any localization knowledge. In fact, to fully understand an environment, in addition to learning the location of specific cues (localization learning), it is necessary to learn how to move in that environment (procedural learning). Procedural components include behaviors such as fear suppression in an open space, leaving peripheral areas, development of efficient explorative strategies and acquisition and utilization of snapshots of the target view and of the representation-forming procedures⁴⁸. Localization and procedural aspects of spatial learning are widely inter-related, even if multiple and differentiated brain areas contribute to them. The most influential theory on spatial learning suggests that *hippocampal areas* have a selective role in high-order components, such as place learning^{49,50,51} or processes underlying the establishment of relational representations⁵². By contrast, hippocampal-independent mechanisms, suggested to be involved in those forms of learning related to procedural components, bring brain structures into play that are primarily involved in sensorimotor functions, such as the cerebellum⁵³.

However, in studies using the novelty test in adult rodents, selective deficits in spatial novelty reactions have been observed following different hippocampal damages^{54,55,56} or in a mouse strain known for hippocampal dysfunction^{57,41}. Furthermore, the septo-hippocampal pathway appears to be specifically involved in the spatial novelty response, as indicated by studies in juvenile and adult rats with basal forebrain cholinergic lesions^{54,56}. Of note is that the formation of the adult-like typical laminar pattern by septal axons in the hippocampal terminal fields occurs around day 14 in rodents, and the period between days 11 and 21 represents the peak time of synaptogenesis in such region^{58,59}. A particularly interesting factor is the spatial working memory. It was believed that the hippocampal system integrity was necessary to learn spatial tasks requiring working memory abilities^{60,49}.

Several recent studies indicate a role for NCAM in learning and establishment of long-term memory^{61,62}. NCAM knock-out mice have shown deficiency in spatial learning when tested in a Morris water maze⁶³. Thus, NCAM is important during brain development and in synaptic plasticity in the adult brain associated with regeneration and learn-

ing⁶⁴. Furthermore, cerebellar mutant mice have been shown to display defective spatial learning and memory in water maze even without significant depletion of cells in the hippocampal formation⁶⁵, emphasizing the importance of the cerebellum in cognitive processes⁶⁶.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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