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Learning and Memory Deficits in Notch Mutant Mice

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Summary

Notch is a critical component of evolutionarily conserved signaling mechanisms that regulate development [1] and may contribute to plasticity-related processes, including changes in neurite structure [2] and maintenance of neural stem cells [3]. Deficits in the Notch pathway are responsible for Alagille [4] and Cadasil syndromes [5], which are associated with mental retardation and dementia. Additionally, in postmitotic neurons, Notch proteins interact with presenilins [6-9] and with β-amyloid precursor protein [10] and could therefore have a role in the memory deficits associated with familial and sporadic Alzheimer's disease. To test if alterations in Notch signaling can lead to learning and memory deficits, we studied mice with mutations in this pathway. Here, we show that null heterozygous mutations in *Notch1* result in deficits in spatial learning and memory without affecting other forms of learning, motor control, or exploratory activity. We also show that null heterozygous mutations in the downstream cofactor *RBP-J* result in similarly specific spatial learning and memory deficits. These data indicate that a constitutive decrease in Notch signaling can result in specific learning and memory deficits and suggest that abnormalities in Notch-dependent transcription may contribute to the cognitive deficits associated with Alzheimer's disease and Alagille and Cadasil syndromes.

Results and Discussion

Besides its critical role in neuronal development, the Notch pathway may also function in the adult brain and may control processes such as neuritic growth [2] and neural stem cell pool maintenance [3], which have been related to cognitive function [11–14]. In postmitotic adult brain neurons, Notch molecules are coexpressed and interact with presenilins (PS), which have been implicated in familial Alzheimer's disease (FAD) [6–9]. PS are a critical component of γ-secretase and are required for the cleavage and nuclear access of the transcriptionally active Notch intracellular domain, and mutations in PS result in altered Notch processing and in a neurogenic phenotype [6, 7, 15, 16]. This PS-dependent cleavage is essential for Notch's role in neurite outgrowth inhibition [17, 18] and maintenance of neural stem cells [3]. Gamma-secretase/PS also cleaves β-amyloid precursor protein (APP), generating β-amyloid in AD [19], and the transcriptionally active APP intracellular domain (AID), which interacts and inhibits the Notch intracellular domain [10, 20, 21]. Inhibitors of γ-secretase have been developed as possible therapeutic strategies for AD. Interestingly, the majority of these inhibitors also downregulate Notch signaling and impair Notch function [18, 22, 23]. Even γ-secretase inhibitors that could specifically target APP cleavage would result in diminished generation of the APP intracellular domain and could alter Notch signaling [10].

To evaluate the effects of decreasing Notch signaling in brain function and more specifically in learning and memory, we used mice carrying a heterozygous null mutation in the *Notch1* gene (*Notch1*$^{+/-}$, homozygotes are lethal [24]). Despite a constitutively hypofunctional Notch pathway, these mice are viable, healthy, and do not have obvious developmental deficits. Although they exhibit a transient premature myelination of small-caliber axons, they have normal myelination and brain architecture in adulthood [25]. In the studies described here, we used animals older than 60 days, an age at which myelination is already normal in these mice [25].

Because *Notch1* is expressed in the hippocampus [2, 8] and deficits in this brain region have been implicated in the cognitive abnormalities associated with AD, we tested the *Notch1*$^{+/-}$ mice in the hidden version of the water maze; performance in this task is known to be sensitive to hippocampal lesions [26]. In this spatial navigation task, animals learn to locate a submerged platform in a pool filled with opaque water. During training, mice were given six trials a day (three sessions of two trials, 30 s intertrial interval [ITI]) for 5 days. Because the time animals take to reach the platform during training is not a very sensitive measure of spatial learning [27], we gave the mice probe trials (days 3 and 5), where the platform was removed from the pool and the mice were allowed to search for it for 60 s. During training, all animals showed an improvement in the time they took to find the platform ($F_{14,378} = 15.2$, $p < 0.05$), and no difference was found between *Notch1*$^{+/-}$ mice and wild-type (WT) littermates ($F_{1,27} = 1.09$, $p > 0.05$; Figure 1A). However, during the first probe trial, given after 3 days of training, there was a significant interaction between genotype and the percentage of time spent in each quadrant of the pool ($F_{3,81} = 3.72$, $p < 0.05$). WT mice searched significantly longer in the quadrant where the platform was during training (training quadrant [TQ])

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than *Notch1*$^{+/-}$ mice ($F_{1,27} = 6.26$, $p < 0.05$; Figure 1C). Additionally, while WT mice searched significantly longer in the TQ than in the other quadrants ($F_{3,56} = 30.6$, $p < 0.05$, posthoc tests Fisher's PLSD, $p < 0.05$), *Notch1*$^{+/-}$ did not ($F_{3,52} = 2.55$, $p > 0.05$; Figure 1C). Using another very sensitive measure of spatial learning [28], we confirmed that WT mice searched on average closer to the exact platform position than to the corresponding positions in the other quadrants of the pool ($F_{3,56} = 17.84$, $p < 0.05$; posthoc, $p < 0.05$), while mutants did not ($F_{3,52} = 1.063$, $p > 0.05$; Figure 1D). No differences in swimming speed (WT = 19.3 ± 0.58, *Notch1*$^{+/-}$ = 19.0 ± 1.10, $F_{1,27} = 0.07$, $p > 0.05$), floating, or thigmotaxic behavior (data not shown) were observed between WT and *Notch1*$^{+/-}$ mice. Altogether, these data demonstrate that the *Notch1*$^{+/-}$ mutation impaired spatial learning.

Importantly, with extended training, *Notch1*$^{+/-}$ mice also learned to search selectively for the missing platform. During a probe trial given after 5 days of training, *Notch1*$^{+/-}$ mutants spent the same amount of time searching in the TQ as did WT mice ($F_{1,27} = 0.05$, $p > 0.05$; Figure 1E). Consistently, both mutants ($F_{3,52} = 6.21$) and WT ($F_{3,56} = 11.8$) searched on average closer to the exact platform position than to the other positions in the pool (posthocs, $p < 0.05$; Figure 1F). *Notch1*$^{+/-}$ mice could remember as well as WT mice the position of the platform for 8 ($F_{1,27} = 1.89$, $p > 0.05$) or 45 days ($F_{1,21} = 0.31$, $p > 0.05$; data not shown).

We also assessed reversal learning and memory in the water maze. Mice were first trained as described above and were then tested for their ability to learn a different platform position. Importantly, to ensure that differences in the initial training in the water maze did not confound reversal testing, we used a group of WT and *Notch1*$^{+/-}$ mice with matched performances at the end of the initial training. These mice were trained to find a new platform position during 12 consecutive trials (30-s intertrial) and were subjected to a probe trial immediately after training (30 s, short term [ST]) or 48 hr later (long term). During training in this novel position, *Notch1*$^{+/-}$ mice learned the new platform position normally ($F_{1,25} = 0.57$, $p > 0.05$; Figure 1G), and they searched significantly longer for the missing platform in the new training quadrant than in the other quadrants ($p < 0.05$), as did WT mice ($p < 0.05$; Figure 1H). However, 48 hr later, *Notch1*$^{+/-}$ mice no longer searched preferentially in the training quadrant, while WT still did ($p < 0.05$ for all quadrants). WT mice searched similarly for the new TQ immediately and 48 hr later ($t_{13} = 2.04$, $p > 0.05$), while *Notch1*$^{+/-}$ mutants searched significantly less in the TQ at 48 hr ($t_{12} = 4.07$, $p < 0.05$). This finding indicates that they forgot the new platform position. Interestingly, 48 hr after reversal training, *Notch1*$^{+/-}$ mutants searched longer in the original training quadrant (marked as t, Figure 1H) than immediately after training ($t_{12} = -3.1$, $p < 0.05$). This finding indicates that, as they forgot the new platform position, they showed a tendency to search in the original training quadrant. Taken together, these data suggest that the *Notch1*$^{+/-}$ mutation impairs the formation of long-term spatial memories.

To determine whether deficits in motivation, visual acuity, or motor coordination could account for the abnormalities in spatial learning, a group of animals was tested in the visible platform version of the water maze; performance in this task is not affected by hippocampal lesions [26]. Both mutant and WT control mice learned this task similarly, since the times taken to reach the visible platform were not different between the groups ($F_{1,10} = 0.63$, $p > 0.05$) (Figure 1B).

Since *Notch1* is expressed in the cerebellum [8] and this structure is known to have a role in motor function, we tested the *Notch1*$^{+/-}$ mutants on a rota-rod, which tests motor coordination and motor learning [29]. We gave the animals five trials (300 s each) with a 5-min intertrial interval on an accelerating rota-rod (4–40 rpm) and measured the time it took for the mice to fall off. All subjects showed an increase in the latency to fall across trials ($F_{1,17} = 13.3$, $p < 0.05$; Figure 2), and no difference was observed between WT and *Notch1*$^{+/-}$ mice in the performance of the task ($F_{1,17} = 4.84$, $p < 0.05$). Memory for this task was intact after 1 day or 20 days, indicating that motor coordination, motor learning, and motor memory were not disrupted in *Notch1*$^{+/-}$ mice (Figure 2).

Next, we tested the specificity of the spatial learning deficits of the *Notch1*$^{+/-}$ mice. *Notch1*$^{+/-}$ mice were normal in a range of other different and dissociable hippocampal-dependent tests [30] (contextual conditioning, contextual discrimination [31], passive avoidance [32], and social recognition [33]). They were also normal in nonhippocampal forms of classical conditioning, such as cued conditioning [31]. *Notch1*$^{+/-}$ mice showed normal exploratory activity in a conditioning chamber [34] and in an open field [29, 35]; normal anxiety when tested in an open field by measuring area occupancy and defecation; and normal muscular coordination (gait), muscular strength (wire hang), body weight, eye blink reflex, reaching and grasping behaviors, and other neurological tests [35] (Table 1). This extensive behavioral analysis demonstrates the specificity of the spatial learning deficits described for the *Notch1*$^{+/-}$ mice, and they indicate that they are not caused by gross neurological abnormalities.

The Notch pathway can signal through a CBF1/RBP-J-dependent pathway (transcriptionally active) or a CBF1/RBP-J-independent pathway [36]. RBP-J is activated by all four Notch receptors (not only by Notch1), and therefore mutations in this transcription factor should impair Notch signaling more severely than *Notch1* mutations [37]. To determine if alterations in the CBF1/RBP-J-dependent pathway affect spatial learning, we tested *RBP-J* null heterozygous mice (*RBP-J*$^{+/-}$, homozygotes die in utero [38]) in the hidden version of the water maze. These mice had a similar genetic background and were trained by using the same procedures as described above for the *Notch1*$^{+/-}$ mutants. During training, all animals showed an improvement in the time taken to find the platform ($F_{14,406} = 16.3$, $p < 0.05$), but there was a significant interaction between session and genotype ($F_{1,14} = 16.3$, $p < 0.05$); *RBP-J*$^{+/-}$ were significantly slower to learn the task than WT littermates ($F_{1,29} = 12.1$, $p > 0.05$; Figure 3A). During the probe trial given after 3 days of training, the WT mice spent significantly more time searching for the platform in the TQ than *RBP-J*$^{+/-}$ mice ($F_{1,29} = 7.13$, $p < 0.05$;

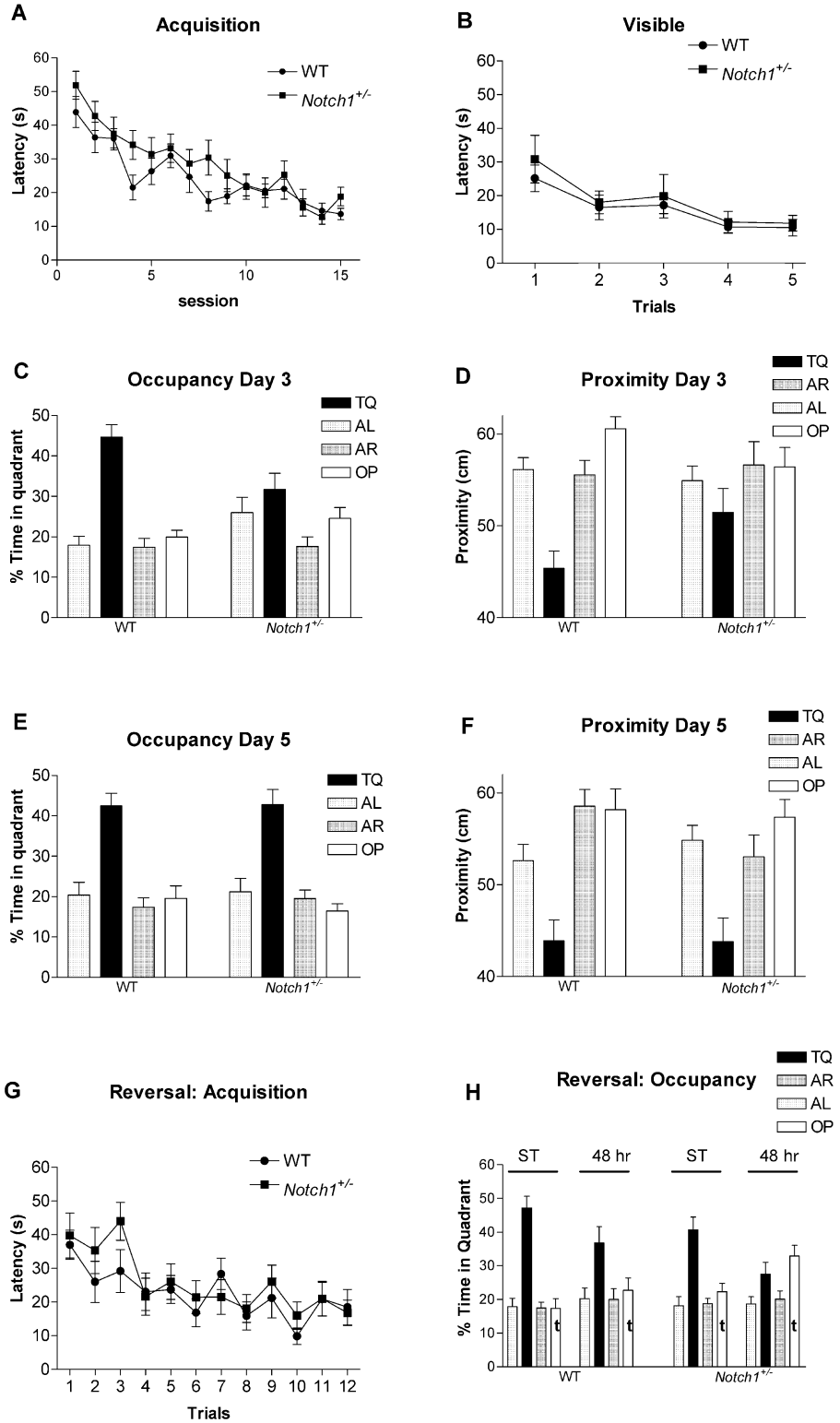

Figure 1. Spatial Learning Impairments in *Notch1*$^{+/-}$ Mice

(A) *Notch1*$^{+/-}$ mice (n = 15) and WT littermates (n = 14) were trained for 5 days, with six trials a day (in sessions of two trials, 30-s intertrials) in the water maze. The average latency to reach the hidden platform is plotted across sessions. Escape latencies decrease with training, and there is no difference in latencies between mutants and controls.

(B) After the hidden version of the water maze, a group of animals was run in the visible platform task, with platform and starting position varying between each trial. There was no difference in latency to get to the platform between WT (n = 6) and mutants (n = 6) across trials.

(C) The results of a probe trial given after 3 days of training. The percentage of time animals spent searching in each of the training quadrants

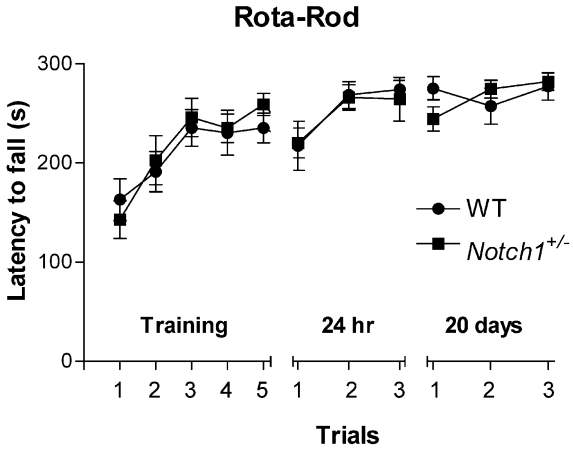

Figure 2. *Notch1*$^{+/-}$ Mutation Does Not Affect Motor Learning or Motor Coordination

Mice were trained for 5 trials (300 s each), with a 5-min ITI on an accelerating rota-rod (4–40 rpm), and the time it took for the mice to fall off was measured. Memory for this task was assessed after 1 day or 20 days (three trials, 5-min ITI). There was no difference between WT (n = 11) and *Notch1*$^{+/-}$ (n = 8) mice in acquisition or memory of the task.

Figure 3C). Furthermore, WT mice searched closer to the exact platform position than to the corresponding positions in the other pool quadrants ($F_{3,56} = 5.63$, $p < 0.05$ for all quadrants); while *RBP-J*$^{+/-}$ littermates did not ($F_{3,60} = 2.75$, $p > 0.05$; Figure 3D). No differences in swimming speed (WT = 20.5 ± 1.13; *RBP-J*$^{+/-}$ = 20.9 ± 0.81, $F_{1,29} = 0.11$, $p > 0.05$), floating, or thigmotaxic behavior (data not shown) were observed between WT and *RBP-J*$^{+/-}$ mice. With additional training, the *RBP-J*$^{+/-}$ mice learned to search selectively for the missing platform ($F_{3,60} = 8.26$, $p < 0.05$, Figure 3E; $F_{3,60} = 6.71$, $p < 0.05$, Figure 3F) and did not significantly differ from WT in time spent searching in TQ ($F_{1,29} = 2.393$, $p > 0.05$, Figure 3E); although, they still searched for considerably less time in TQ than WT (35.6 ± 2.75 versus 41.8 ± 2.87). Because of this apparent difference in performance even after overtraining, we did not perform a reversal training task, since we could not ensure that differences in the initial water maze training would not confound the reversal testing. The spatial learning deficits observed in the *RBP-J*$^{+/-}$ mice did not arise from motivation, motor coordination, or visual acuity problems since both mutant and WT mice learned the visible task similarly ($F_{1,13} = 0.35$, $p > 0.05$; Figure 3B). Also, the phenotype of the *RBP-J*$^{+/-}$ mice seemed more severe than that of the *Notch1*$^{+/-}$ mice. The *RBP-J*$^{+/-}$ mice had impairments that were already apparent during the acquisition of the task, while *Notch1*$^{+/-}$ mice did not, a finding that is consistent with the hypothesis that RBP-J-dependent signaling of all Notch receptors is compromised in these mice.

Similarly to *Notch1*$^{+/-}$ mice, the spatial learning deficits of the *RBP-J*$^{+/-}$ mice were very specific. Other hippocampal-dependent tasks, such as contextual conditioning (24 hr: 28.8 ± 7.86 versus 29.7 ± 6.18; 7 days: 20.9 ± 5.49 versus 16.2 ± 4.89), were indistinguishable between WT and *RBP-J*$^{+/-}$, even when using a very sensitive protocol to assess hippocampal function [39], with only a 20-s interval between placement in the context and delivery of the shock (24.6 ± 6.44 versus 24.5 ± 7.1). Also, nonhippocampal forms of pavlovian conditioning, such as tone conditioning (41.7 ± 9.44 versus 36.8 ± 8.85), anxiety and baseline exploratory activity (32.9 ± 6.35 versus 35.4 ± 4.96), and a variety of neurological measures assessing muscular coordination, muscular strength, eye blink reflex, and reaching and grasping (data not shown), were also indistinguishable between WT and *RBP-J*$^{+/-}$ mice.

The results presented here demonstrate that a chronic decrease in Notch1 function results in spatial learning deficits and suggest that Notch-dependent transcription is critical for spatial learning. The exquisite specificity of these learning and memory deficits argues against the possibility that the mutations studied result in gross developmental abnormalities that could account for the learning deficits. Also, we observed long-term spatial memory deficits in experiments in which *Notch* mutant mice had normal acquisition and short-term spatial memory. This strongly argues against a general deficit in hippocampal information processing and points toward a specific functional deficit due to changes in Notch signaling. It is important to notice that previous studies have shown that different hippocampal-dependent functions are differentially affected by hippocampal manipulations [30, 40]. Our results with heterozygotes indicate that Notch mutations affect a specific domain of hippocampal function that is especially important for spatial learning and memory, but it is possible that com-

is shown. WT mice searched selectively since they spent significantly more time searching in the training quadrant (TQ, black bars) than in any of the other quadrants, while *Notch1*$^{+/-}$ mutants did not search selectively in any of the quadrants (adjacent left [AL], adjacent right [AR], and opposite [OP], white and shaded bars). Also, WT mice spent significantly more time searching in the training quadrant than *Notch1*$^{+/-}$ mice (44.6 ± 3.15 versus 31.7 ± 4.08).

(D) A different measure to assess selective searching during the same day 3 probe trial. WT mice searched on average closer to the exact position of the platform during training (TQ, black bars) than to the corresponding positions in the other quadrants, while mutants did not.

(E) A probe trial given after 5 days of training. Both mutants spent significantly more time searching in the training quadrant than in any of the other quadrants, and they spent similar time searching in the training quadrant.

(F) Average proximity during the same day 5 probe trial. Both mutants and WT searched on average closer to the exact platform position than to the other positions in the pool.

(G) Acquisition of a new platform position during reversal learning (12 consecutive trials, 30-s ITI). No differences between *Notch1*$^{+/-}$ (n = 13) and WT (n = 14) mice were observed in reversal acquisition.

(H) During a probe trial given 30 s (short term [ST]) after reversal training, both *Notch1*$^{+/-}$ mutants and WT mice searched longer in the TQ than in the any of the other quadrants. WT mice remembered the new platform position for at least 48 hr, while *Notch1*$^{+/-}$ mice did not. Interestingly, *Notch1*$^{+/-}$ mutants searched longer in the original training quadrant (marked with a t) 48 hr after reversal training than immediately after training.

Table 1. Specificity of the *Notch1*$^{+/-}$ Mice Deficits

Task	WT	*Notch1*$^{+/-}$
Activity in chamber	34.5 ± 7.11	31.6 ± 6.81
Contextual conditioning (% freezing, 24 hr)	35.0 ± 5.51	35.1 ± 6.93
Tone conditioning (% freezing)	22.0 ± 11.1	30.2 ± 8.80
Contextual discrimination (discrimination ratio: freezing chamber A/A + B)	0.62 ± 0.04	0.61 ± 0.07
Passive avoidance (48 hr, latency to enter dark chamber, s)	402 ± 71.7	406 ± 60.2
Social recognition (recognition ratio: initial/initial + 24 hr)	0.41 ± 0.04	0.40 ± 0.05
Open field (path length, cm)	2664 ± 411	2762 ± 513
Open field (outer/inner + outer zone %)	97.6 ± 0.74	96.8 ± 0.91
Open field (defecation, number of boli)	1.6 ± 0.67	1 ± 0.45
Wire hang (latency to fall, s)	27.2 ± 3.18	25.4 ± 3.04
Ambulance (right gait, cm)	5.94 ± 0.31	5.95 ± 0.44
Ambulance (left gait, cm)	6.21 ± 0.25	6.06 ± 0.24
Body weight (g)	24.8 ± 1.71	25.0 ± 1.18

Other hippocampal functions different than spatial navigation, such as place (contextual conditioning, contextual discrimination, and passive avoidance) and social recognition, and nonhippocampal functions, such as cued conditioning, are unaffected in *Notch1* mutants. Exploratory activity (chamber and open field activity) and anxiety (open field occupancy and defecation) are also normal. Muscular coordination (gait) and muscular strength (wire hang) are unaffected.

plete deletions would result in more generalized deficits. Also, it is conceivable that very specific and undetected neuroanatomical changes taking place during development account for the specific spatial learning deficits of the *RBP-J*$^{+/-}$ and *Notch1*$^{+/-}$ mice. Even if this would be the case, the findings presented here would still be important for understanding the deficits associated with Alagille and Cadasil syndromes and Alzheimer's disease. However, in agreement with our conclusions, reducing Notch function specifically in adult *Drosophila* leads to progressive neurological dysfunction [41].

The results presented here also have important implications for the development of therapeutic strategies for AD. Moreover, they may help us to understand the mechanisms associated with the generation of cognitive deficits in AD. Both increased PS-dependent cleavage of Notch in FAD and increased APP signaling trough AID in sporadic AD [7, 10] can result in altered Notch signaling. It is possible that the synaptic loss and the synaptic dysfunction observed in AD [42], which correlate with the cognitive deficits, could result from an interaction between the effects of extracellular soluble β-amyloid and intracellular alterations in Notch and APP signaling [21, 43].

In conclusion, our results are consistent with the hypothesis that Notch signaling is involved in learning and memory processes in the adult brain, and they suggest that abnormalities in Notch-dependent transcription may contribute to the learning and memory deficits associated with Alzheimer's disease and Alagille and Cadasil syndromes.

Experimental Procedures

Animals

The generation of the different genetically modified mice was described before [24, 38]. The *Notch1*$^{+/-}$ population was backcrossed eight generations into the C57BL/6J background from the original genetic background. The *RBP-J*$^{+/-}$ population was backcrossed 13 generations into the C57BL/6N background from the original genetic background. In every experiment, we used isogenic littermates, except for the genes of interest. Genotyping was done by PCR. All experiments were done blind with respect to genotype and were conducted with the approval of the UCLA Animal Research Committee of the Chancellor's Office of Protection of Research.

Behavioral Tasks

The basic water maze protocols and equipment are described elsewhere [29]. Each water maze experiment was replicated three times with different groups of about six mutants and six WT littermates. The contextual conditioning (2 s, 0.75 mA, 150 s placement to shock interval, unless indicated otherwise), tone conditioning (30 s, 85 dB, 2.8 kHz tone coterminating with the shock), and contextual discrimination experiments were performed as previously described [29, 31] and by using computer-assisted assessment of freezing [34]. Passive avoidance was performed as previously described [32]: a 2-s 0.75 mA shock was delivered after the animal completely entered the dark chamber and the communication door was closed, and a 600 s test period occurred 24 hr later. The social recognition task was performed according to [33], and the roto-rod and other motor and exploratory tasks were performed as described in [29].

Statistical Analysis

A two-way ANOVA with repeated measures was used to analyze the acquisition data from the water maze and rota-rod tasks and to investigate possible interactions between quadrant occupancy and genotype during probe trials. Single-factor ANOVA was used to analyze the effect of the quadrant in both occupancy and proximity, and posthoc comparisons (Fisher's PLSD) between quadrants were performed when there was an effect of quadrant. Comparison between mutants and WT for all the other measures described (including TQ occupancy) was also done by using single-factor ANOVA.

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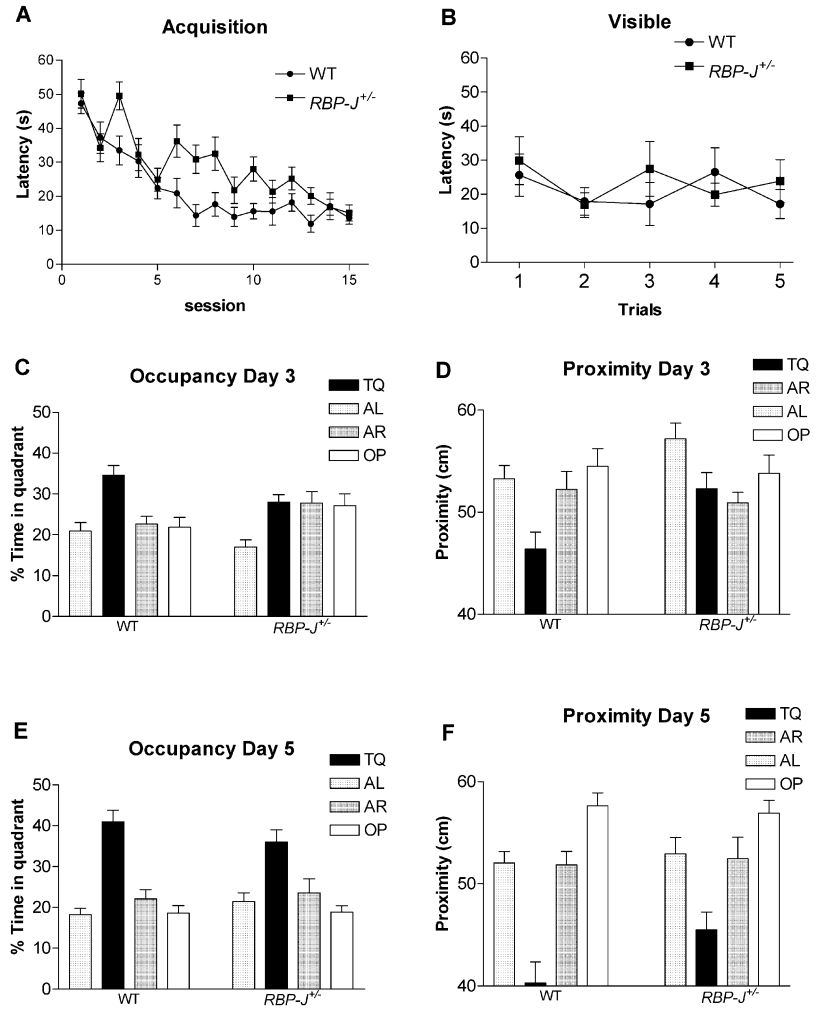

Figure 3. Spatial Learning Deficits in *RBP-J*$^{+/-}$ Mice

(A) *RBP-J*$^{+/-}$ mice (n = 16) and WT littermates (n = 15) were trained for 5 days, as described for the *Notch1*$^{+/}$ mice. The average latency to reach the hidden platform is plotted across sessions. Escape latencies decrease with training, but there is a significant interaction between genotype and training across sessions. *RBP-J*$^{+/-}$ mice are slower than WT to learn the hidden watermaze task.

(B) After the hidden version of the water maze, a group of animals was run in the visible platform task, with platform and starting position varying between each trial. In the visible version of the water maze, there was no difference in latency to get to the platform between WT (n = 8) and mutants (n = 7) across trials.

(C) The results of a probe trial given after 3 days of training (TQ, black bars; adjacent left [AL], adjacent right [AR], opposite [OP], white and shaded bars). The percentage of time animals spent searching in each of the training quadrants is shown. *RBP-J*$^{+/-}$ mice spent significantly less time searching in the training quadrant than WT mice (27.6 ± 1.75 versus 35.4 ± 2.37).

(D) During the same day 3 probe trial, WT mice searched on average closer to the exact position of the platform during training than to the corresponding positions in the other quadrants, while mutants did not.

(E) A probe trial given after 5 days of training. Both mutants and WT searched selectively and spent a similar amount of time searching in the training quadrant.

(F) Proximity during the day 5 probe trial. Both mutants and WT searched on average closer to the exact platform position than to the other corresponding positions in the pool.

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