

pathway and independent of follicularly organized lymphoid tissue perhaps offers a glimpse at the primitive, specific antibody-dependent immune system.

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Impaired Cued and Contextual Memory in NPAS2-Deficient Mice

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Neuronal PAS domain protein 2 (NPAS2) is a basic helix-loop-helix (bHLH) PAS domain transcription factor expressed in multiple regions of the vertebrate brain. Targeted insertion of a β -galactosidase reporter gene (*lacZ*) resulted in the production of an NPAS2-*lacZ* fusion protein and an altered form of NPAS2 lacking the bHLH domain. The neuroanatomical expression pattern of NPAS2-*lacZ* was temporally and spatially coincident with formation of the mature frontal association/limbic forebrain pathway. NPAS2-deficient mice were subjected to a series of behavioral tests and were found to exhibit deficits in the long-term memory arm of the cued and contextual fear task. Thus, NPAS2 may serve a dedicated regulatory role in the acquisition of specific types of memory.

Because pharmacological inhibitors of gene expression impede learning in a variety of experimental paradigms (1), it is anticipated that gene-specific transcription factors may play a regulatory role in learning and memory. For example, mice deficient in cyclic adenosine 3',5'-monophosphate (cAMP) response element-binding protein exhibit normal learning and short-term memory but are

deficient in long-term memory (2). The calcium- and cAMP-mediated signal transduction pathways, as well as the transcription factors that alter gene expression as a terminal result of intracellular signaling, are expressed in a wide spectrum of invertebrate and vertebrate cell types (3). It is logical to assume that ubiquitous signaling pathways facilitate stimulus-induced changes in neuronal gene expression. Less obvious is how a selective regional pattern of regulatory response may be orchestrated.

The onset of neuronal PAS domain protein 2 (NPAS2) gene expression occurs within the first week of postnatal development, is exclusively restricted to neurons, and is distributed within a stereotypic pattern of fore-

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brain nuclei (4). This expression pattern is temporally matched with the ontogeny of learning and memory (5) and spatially matched with the frontal association/limbic forebrain pathway (6). A targeted disruption of the NPAS2 allele was generated in 129S6/SvEvTac-derived embryonic stem cells (Fig. 1, A and B) such that the coding exon for the basic helix-loop-helix (bHLH) domain was replaced with a modified lacZ gene from *Escherichia coli* (7). As shown in Fig. 1C, the NPAS2-lacZ allele produces two distinct mRNA products. The first and anticipated mRNA splices the first coding exon of NPAS2 to the second coding exon fused to the lacZ gene. The second transcript skips the second coding exon (containing the inserted lacZ gene and the downstream polyA-neo cassette) and instead splices the first coding exon of NPAS2 onto the third coding exon. Western blot analysis revealed that the variant NPAS2 transcript produced in NPAS2-lacZ mice encoded an altered form of NPAS2 lacking the bHLH domain (Δ bHLH-NPAS2) (Fig. 2, A and B). A transient transfection assay was used to assess the functional activities of native NPAS2 and NPAS2- Δ bHLH (8) as tested with and without the obligate heterodimeric partner of NPAS2, brain and muscle ARNT (aryl hydrocarbon nuclear translocator)-like (BMAL) protein (9, 10). Substantial, BMAL-dependent transcriptional activation was only observed in cells transfected with the expression vector encoding wild-type NPAS2 (Fig. 2C). By contrast, the expression vector encoding the NPAS2- Δ bHLH variant exhibited minimal induction of the NPAS2-dependent reporter gene in comparison to the BMAL expression vector alone.

We next defined the neuroanatomical expression pattern of β -galactosidase (β -Gal)

activity in NPAS2-lacZ mice (11). NPAS2-lacZ expression was observed in the cortex, hippocampus, striatum, amygdala, and thalamus, but not in the cerebellum or brainstem of NPAS2-lacZ homozygous ($-/-$) mice (Fig. 3, A through F). Particularly intense

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Fig. 2. (A) Schematic diagrams showing splicing patterns observed for NPAS2 mRNA. NPAS2 mRNA in wild-type ($+/+$) mice splices the first coding exon onto the second (bHLH-encoding) exon. An altered form of NPAS2 mRNA (NPAS2- Δ bHLH) is produced in NPAS2-lacZ ($-/-$) mice where splicing of the first coding exon of the NPAS2 gene onto the third coding exon occurs. If translation of the spliced form of NPAS2 in NPAS2-lacZ mice occurs at the second available methionine (M_2), a variant form of NPAS2 is produced, containing six aberrant residues encoded by the first coding exon, no part of the bHLH domain, yet all remaining sequences of the intact NPAS2 protein beginning with coding exon 3 (37). **(B)** Western blot assays of native NPAS2 and a bHLH-deleted form of NPAS2 (NPAS2- Δ bHLH). An immunoreactive species specific for wild-type NPAS2 was found in (i) 293 cells transfected with

the expression vector encoding native NPAS2 and in (iii) brain protein lysates from ($+/+$) mice. An immunoreactive species ~ 10 kD smaller than native NPAS2 was found in (ii) 293 cells transfected with the expression vector encoding NPAS2- Δ bHLH as well as in (iv) brain protein samples derived from NPAS2-lacZ ($-/-$) mice. **(C)** Transient transfection assays of native and Δ bHLH forms of NPAS2. Cultured 293 cells were transfected with an NPAS2-responsive reporter gene along with half-log increasing increments (1, 3, and 10 ng) of expression vectors encoding BMAL, native NPAS2 (WT NPAS2), NPAS2- Δ bHLH (Δ bHLH), or combinations thereof. Significant, dose-dependent increases in reporter gene activity were only observed in cells cotransfected with both the BMAL and native NPAS2 expression vectors.

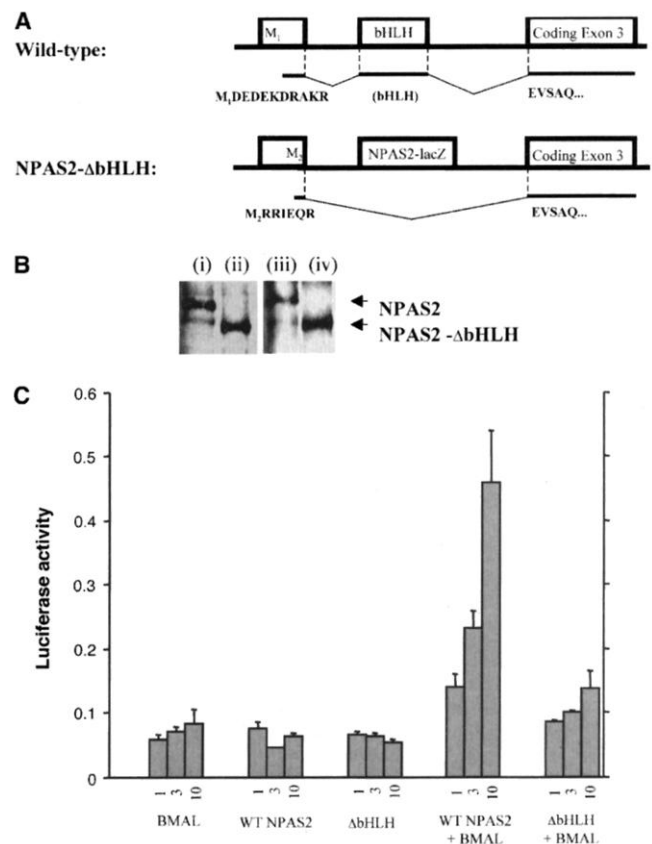
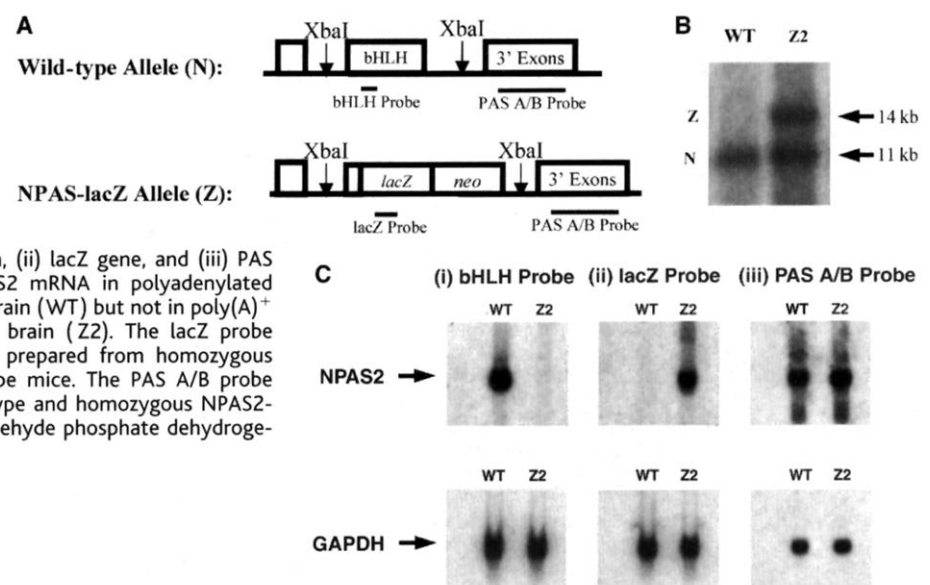


Fig. 1. Generation of NPAS2-lacZ mice. (A) Schematic diagram of genomic mouse DNA in the region surrounding the bHLH-encoding exon of the endogenous NPAS2 gene (N) or the disrupted NPAS2-lacZ allele (Z). **(B)** Southern blot analysis indicating the 11-kb Xba I wild-type NPAS2 allele (N) and the 14-kb Xba I fragment specific to the targeted allele (Z) in either wild-type (WT) or homozygous NPAS2-lacZ (Z2) mice. **(C)** Northern blot analysis with probes derived from the (i) bHLH-encoding exon, (ii) lacZ gene, and (iii) PAS domain. The bHLH probe detected intact NPAS2 mRNA in polyadenylated [poly(A)⁺] RNA prepared from wild-type mouse brain (WT) but not in poly(A)⁺ prepared from homozygous NPAS2-lacZ mouse brain (Z2). The lacZ probe detected an NPAS2-lacZ fusion mRNA in RNA prepared from homozygous NPAS2-lacZ mice but not in RNA from wild-type mice. The PAS A/B probe detected similarly sized mRNA from both wild-type and homozygous NPAS2-lacZ mice. The filters were reprobed for glyceraldehyde phosphate dehydrogenase (GAPDH) for comparison.



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β -Gal staining was observed in the barrelfields (Fig. 3, G and H), somatosensory cortical structures implicated in the processing of complex sensory information gathered from vibrissae (12). NPAS2-lacZ expression in the barrelfields was coincident with cytochrome oxidase staining (13), a histological marker for barrelfield structures (14). NPAS2-lacZ expression was highly enriched

in the brain yet absent from both the supra-chiasmatic nucleus (SCN) and pineal gland (15). Morphological studies revealed no changes in NPAS2-lacZ ($-/-$) animals relative to NPAS2-lacZ heterozygous ($+/-$) or wild-type ($+/+$) animals as assessed by lacZ staining or by gross anatomical examination.

The NPAS2-lacZ ($-/-$) mice were fertile, active, and morphologically indistin-

guishable from NPAS2-lacZ ($+/-$) or ($+/+$) littermates. Male mice generated from F_1 or F_2 mating pairs were tested in a neurobehavioral test battery (16). No statistically significant differences were observed between NPAS2-lacZ ($-/-$) and ($+/+$) littermates in any behavioral assay except for the cued and contextual fear (CCF) task (Fig. 4) (17). In this assay, mice were trained repeatedly with a mild electrical foot shock that occurred immediately after an auditory cue (18) and were subsequently scored for fear behavior (freezing). For contextual memory, mice were tested in the same environment in which they were trained. For cued memory, freezing was assessed in a novel environment, first in the absence of and then in the presence of the training auditory cue.

The NPAS2-lacZ ($-/-$) mice froze less frequently than the ($+/+$) littermates (35% versus 50%) when assayed in the 24-hour contextual arm of the CCF assay (Fig. 4A). There were no differences in freezing behavior between NPAS2-lacZ ($-/-$) and ($+/+$) mice in the 0.5-hour contextual assay, indicating the NPAS2-lacZ ($-/-$) mice were not deficient in short-term memory (Fig. 4B). When assayed in the 24-hour cued arm, NPAS2-lacZ ($-/-$) mice again exhibited a distinct, statistically significant deficit relative to ($+/+$) littermates (40% versus 50%) (Fig. 4C). Before the auditory cue, the freezing behavior of NPAS2-lacZ ($-/-$) mice was similar to that of ($+/+$) littermates.

Having observed intense expression of NPAS2 in the barrelfields, we performed an adaptation of the CCF assay to assess the contribution of tactile information to contextual memory. In the adapted CCF assay, mice were tested 24 hours after training in an environment where smell and appearance were novel yet the texture of the cage flooring was identical to that used in training. Tactile information alone was sufficient to reveal differences in freezing behavior between NPAS2-lacZ ($-/-$) and ($+/+$) mice (12% versus 25%) (Fig. 4D). Although the percentage of freezing time was lower than that observed for either of the classical arms of the test, significant differences were observed between NPAS2-lacZ ($-/-$) mice and their ($+/+$) littermates. Finally, the cued stimulus, when administered in this "tactile only" contextual environment, facilitated recall for both NPAS2-lacZ ($-/-$) and ($+/+$) mice (45% versus 62%).

The results of these behavioral studies indicate that NPAS2-lacZ ($-/-$) mice are deficient in complex emotional long-term memory (CCF task) but not in non-emotional memory (Morris water maze), anxiety (open field, light/dark conflict, and elevated plus maze), or simple aversive conditioning tasks (passive avoidance and step-down avoidance). Likewise, the NPAS2-lacZ ($-/-$)

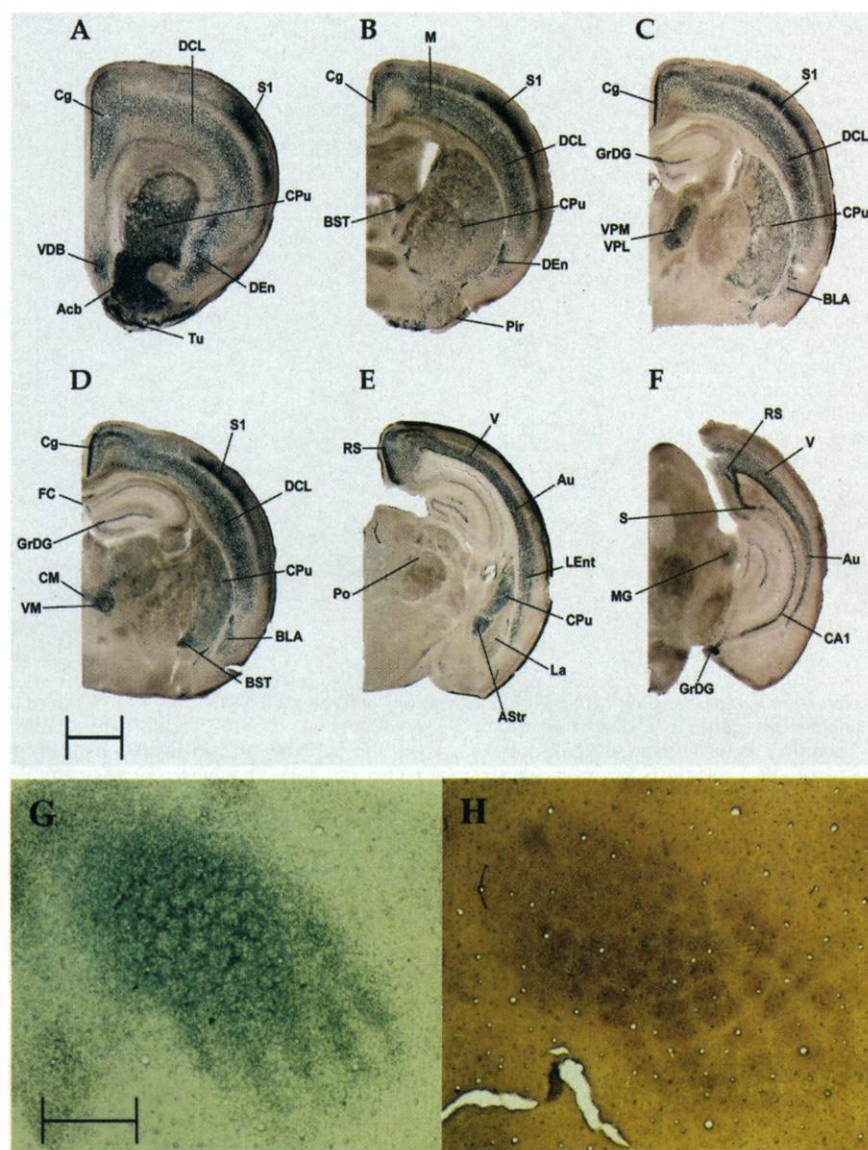


Fig. 3. β -Gal expression pattern in brain tissue of NPAS2-lacZ mice. (A through F) Light microscopic photographs of coronal vibratome brain sections (100 μ m) of 1-month-old male NPAS2-lacZ ($-/-$) mice, stained to reveal β -Gal activity. Sections (A) through (F) correlate to plate numbers 18, 31, 42, 45, 50, and 58, respectively, of (32). [Abbreviations: Acb, accumbens nucleus; AStr, amygdalo-striatal transition area; Au, auditory cortex; BLA, basolateral amygdaloid nucleus; BST, bed nucleus of stria terminalis; CA1, field of hippocampus CA1; Cg, cingulate cortex; CM, centromedian thalamic nucleus; CPu, caudate-putamen (striatum); DCL, deep cortical layers; DEN, dorsal endopiriform nucleus; FC, fasciola cinereum; GrDG, granular layer, dentate gyrus; La, lateral amygdaloid nucleus; LEnt, lateral entorhinal cortex; M, primary and secondary motor cortex; MG, medial geniculate; Pir, piriform cortex; Po, posterior thalamic nucleus; RS, retrosplenial cortex; S, subiculum; S1, primary somatosensory cortex; Tu, olfactory tubercle; V, visual cortex; VDB, ventral limb diagonal band; VM, ventromedial thalamic nucleus; VPL, ventroposteriolateral thalamic nucleus; and VPM, ventroposteromedial thalamic nucleus.] Light microscopic photographs of tangential vibratome brain sections (100 μ m) of 1-month-old male NPAS2-lacZ ($-/-$) mice, stained to reveal (G) β -Gal activity or (H) cytochrome oxidase activity. Scale bars indicate 1 mm.

mice showed no obvious deficits in their ability to perceive and process primary sensory stimuli relating to touch, reflex, balance, vision, or hearing. With respect to sensory information perception, the NPAS2-lacZ ($-/-$) mice and their $(+/+)$ littermates exhibited similar performances in the shock threshold and hot-plate analgesia tests (supplementary data are available at www.sciencemag.org/feature/data/1049880.shl). Thus, NPAS2 appears to be required for the processing of complex sensory information.

Localized regions of the frontal cortex/limbic cortex have been implicated in emotional learning and memory (19). The observed deficits in cued as well as contextual memory suggest that the abnormalities in NPAS2-lacZ ($-/-$) mice include amygdalar-processed information (20, 21). The CCF assay has been described as assessing hippocampal-independent (cued CCF) versus hippocampal-dependent (contextual CCF) learning. Several reports describe hippocampal-lesioned rodents that are primarily defective in contextual but not cued fear (21, 22). In addition, entorhinal-lesioned animals that are defective in contextual learning have been studied (23). We have not identified what anatomical site(s) are responsible for the failure of NPAS2-lacZ ($-/-$) mice to learn in the CCF task. Furthermore, genetic ablation experiments as described herein may differ substantially from lesion studies because related gene product(s) may compensate for a specific genetic deficiency such that functional deficits may occur in only a subset of anatomical sites. However, localization of a deficit within the frontal association/limbic forebrain pathway represents an appealing hypothesis for the NPAS2-lacZ ($-/-$) mice because the thalamo-cortico-amygdalo path-

way is essential for complex emotional memory (24).

Using quantitative trait loci analysis, another study identified, through the examination of the progeny resulting from the cross of two inbred strains, candidate regions encoding factors that influence CCF (25). The region with the strongest influence on CCF learning was found in close proximity to NPAS2. It is possible that a 129S6/SvEvTac-derived gene closely linked to the NPAS2 locus is instead responsible for the observed phenotype. However, previous studies have shown that 129S6/SvEvTac mice respond to this test with an increased level of context-cued freezing relative to C57BL/6J, precisely the opposite trend from what we have observed for NPAS2-lacZ ($-/-$) mice (26).

The closest relative of NPAS2 is CLOCK, a master regulator of circadian rhythm (27). Both NPAS2 and CLOCK function optimally by using the same heterodimeric partner, BMAL, whose temporal expression pattern confers circadian rhythmicity to CLOCK-mediated gene expression in mice (9, 28). Therefore, NPAS2-mediated gene expression may interface with CLOCK regulatory circuits as supported by observations that conditioned fear affects the modulation of circadian rhythms (29).

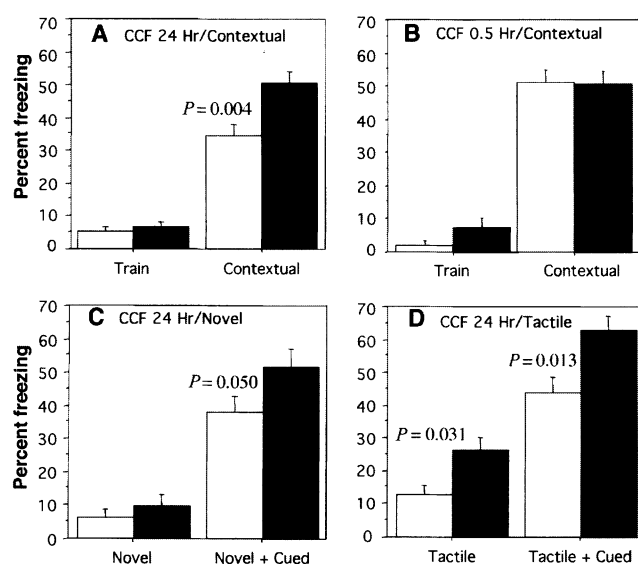
NPAS2-lacZ ($-/-$) mice may have impaired brain function. We hypothesize that NPAS2 gene expression may be activated subsequent to the wiring of neuronal circuits required for learned behavior (30). Once activated, NPAS2 may serve to regulate the neuronal expression of a battery of genes required for the consolidation of long-term memory and/or to maintain a functional relation between multiple components of the frontal association/limbic forebrain pathway.

If NPAS2 indeed proves to function as part of a circadian oscillator that is widespread throughout the forebrain, it is possible that the behavioral deficits observed in the present study are reflective of the importance of rhythmic gene expression on the execution of complex cognitive tasks.

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Fig. 4. CCF behavior in $(+/+)$ and NPAS2-lacZ ($-/-$) mice. Percent freezing of $(+/+)$ (solid boxes) and NPAS2-lacZ ($-/-$) (open boxes) mice in the CCF task (0.5 mA, unconditioned stimulus) for (A) training and 24-hour contextual ($n = 45$ per group), (B) training and 0.5-hour contextual ($n = 41$ per group), (C) novel and novel + cued ($n = 20$ per group), and (D) tactile and tactile + cued ($n = 25$ per group) assays. NPAS2-lacZ ($-/-$) mice (Z2) exhibited statistically significant differences from wild-type littermates (WT) in (A) 24-hour contextual, (C) novel + cued, (D) and tactile and tactile + cued assays. Error bars indicate 95% confidence intervals.



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2 (training), the mice were placed in the same apparatus 6 hours after part 1 and were allowed to explore for 3 min. The conditioned stimulus, 30 s of 80 dB white noise, followed and ended with the unconditioned stimulus, a 1-s 0.5-mA shock. This was followed by a 30-s observation interval. The conditioned stimulus–unconditioned stimulus pairing was repeated for a total of three times, making the entire test duration 6 min. In part 3, the mice were returned to the identical testing apparatus 0.5 hour [CCF 0.5 hour/contextual (Fig. 4B)] or 24 hours [CCF 24 hours/contextual (Fig. 4A)] after the training period for a 3-min assessment of contextual learning. For 24-hour cued memory, the animals were assessed for freezing behavior 45 min after part 3, first in the absence then in the presence of the acoustic cued stimulus. The subjects from part 3 were split into two groups so that testing for cued memory was either in a novel environment [novel versus novel + cued (Fig. 4C)] or in a tactile environment [tactile versus tactile + cued (Fig. 4D)]. The novel environment was an identical apparatus as used in part 1, except it was painted black (versus unpainted aluminum), scented with 0.1% acetic acid (versus ethanol), and contained a black Plexiglas (acrylic plastic) floor insert. The tactile environment was identical, except that the black Plexiglas floor insert was omitted, thereby retaining the wire grid flooring used in the training session. The mice were allowed to explore the novel or tactile chamber for 3 min followed by another 3-min period in the presence of the acoustic stimulus (cued). During each test phase, the animals were scored at 10-s intervals by two independent observers for freezing defined as total immobilization except for respiratory movement. The total number of freezes for each individual according to session was generated, and then, a mean was calculated according to genotype

for a summary measure. Comparisons were made by planned comparisons (StatView, SAS Institute, Cary, NC). For analysis of 24-hour contextual learning (Fig. 4A) or 0.5-hour contextual learning (Fig. 4B), the number of freezes for the 3-min contextual interval (contextual) was compared to the 3-min exploration period during training (train). For analysis of the cued portion, the number of freezes during the 3-min interval preceding the acoustic stimuli [novel (Fig. 4C) or tactile (Fig. 4D)] was compared to the number of freezes during the 3-min acoustic stimulus interval [novel + cued (Fig. 4C) or tactile + cued (Fig. 4D)]. The indicated CCF findings were significant for rejection of the null hypothesis at $P \leq 0.05$ with either parametric or nonparametric stochastic comparisons. Error bars indicate 95% confidence intervals. Similar results were obtained from both observers. The data from one observer are shown in Fig. 4.

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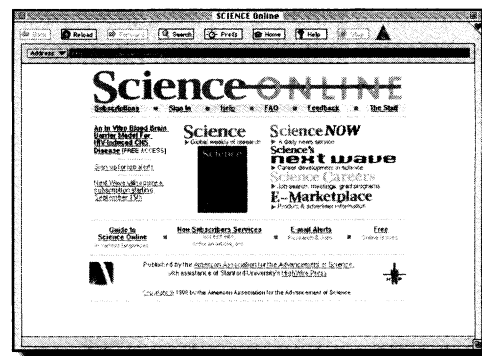
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31. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; I, Ile; K, Lys; M, Met; Q, Gln; R, Arg; S, Ser; and V, Val.
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