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Imaging Physiologic Dysfunction Neurotechnique of Individual Hippocampal Subregions in Humans and Genetically Modified Mice

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Summary

We have developed a variant of functional magnetic resonance imaging (fMRI) designed to be sensitive to static neuronal function. This method is based on resting instead of dynamic changes in oxygen-dependent signal and therefore allows for a spatial resolution that can detect signal from different hippocampal subregions in human subjects as well as in mice. We found that hippocampal signal was significantly diminished in elderly subjects with memory decline compared to age-matched controls, and different subjects showed dysfunction in different subregions. Among healthy elders, signal intensity from the subiculum was correlated selectively with memory performance. This method does not require an activation task; it can be used in anesthetized normal and in genetically modified and cognitively impaired mice. In mice the signal was found to be sufficiently sensitive to detect functional changes in the absence of underlying anatomical changes.

Introduction

A major advance in cognitive neuroscience has come from the development of structural and functional imaging that allows visualization of specific brain regions in behaving human subjects. Recently, it has become possible to carry out functional imaging experiments in nonhuman primates (Logothetis et al., 1999). This research strategy should allow studies in which human and nonhuman primates perform similar behaviors. A parallel strategy of this sort encourages the analysis of imaging experiments to move beyond obtaining correlations between a behavior and a signal in a given region of the human brain to doing intervention experiments in nonhuman primates that are designed to examine the causal relationships between a signal in a brain region and the behavior under study. Still lacking in this repertory of imaging methodologies, however, has been a technique that would extend these comparisons to mice with genetic lesions. To overcome this limitation, we have developed an approach, applicable to both mice and human subjects, based on nontask-related magnetic resonance imaging that relies on a resting rather than dynamic signal.

The current magnetic resonance imaging experiments are based on the principle that T2* (magnetic susceptibility) is sensitive to the ratio of oxyhemoglobin to deoxyhemoglobin (Ogawa et al., 1990), which is an indirect correlate of neuronal function. Since functional MRI (fMRI) first was developed in the early 1990s, nearly all studies have focused on the dynamic shift in T2* signal associated with an activation task, and the success of this analysis has led to the remarkable expansion in the use of fMRI in cognitive neuroscience. Task-induced measurements of dynamic T2* signal introduces several limitations, however. Detecting changes in the T2* signal requires MRI techniques that use rapidly shifting magnetic gradients designed to enhance temporal resolution. The enhanced temporal resolution obtained with these methods comes at the expense of a reduction in spatial and anatomical resolution (Frahm et al., 1993). As a result, dynamic fMRI only has a spatial resolution of a few millimeters, and this resolution is insufficient for evaluating many brain structures such as the subregions of the hippocampus (Stern and Hasselmo, 1999).

In addition to restricted anatomical resolution, other limitations are introduced by requiring subjects to perform an activation task. In certain brain diseases, subjects cannot perform the required task. In patients with Alzheimer's disease, for example, one can only use an activation task to evaluate patients with mild disease (Small et al., 1999), since patients with moderate to severe dementia cannot understand the experimental instructions. Activation tasks are even more limiting in studies of small animals where anesthesia is needed typically to prevent motion-related artifacts.

To overcome the limitations imposed by a task-oriented dynamic T2* signal, we have turned to examining nontask-oriented experiments based on resting T2* signal. The idea of using resting T2* signal derived from the consideration that most causes of brain dysfunction produce changes not only in the active but also in the resting function of neurons. Electroencephalography (EEG) recordings have shown that the resting firing patterns of neuronal populations are changed in many neurological diseases and can occur in the absence of structural damage. More recent studies with positron emission tomography (PET) (for example, Eberling et al., 1997; Small et al., 2000) have extended these observations by showing that changes in resting neuronal metabolism occurs with relatively subtle changes in brain function. Changes in resting neuronal function are correlated with resting oxyhemoglobin concentrations as shown in a study where EEG measurements where coupled with near-infrared spectroscopy (Hoshi et al., 1998). Similar studies have shown a disproportionate change in oxyhemoglobin in the setting of brain disease (Fallgatter et al., 1997). Because resting T2* signal is known to be sensitive to resting oxyhemoglobin levels in blood

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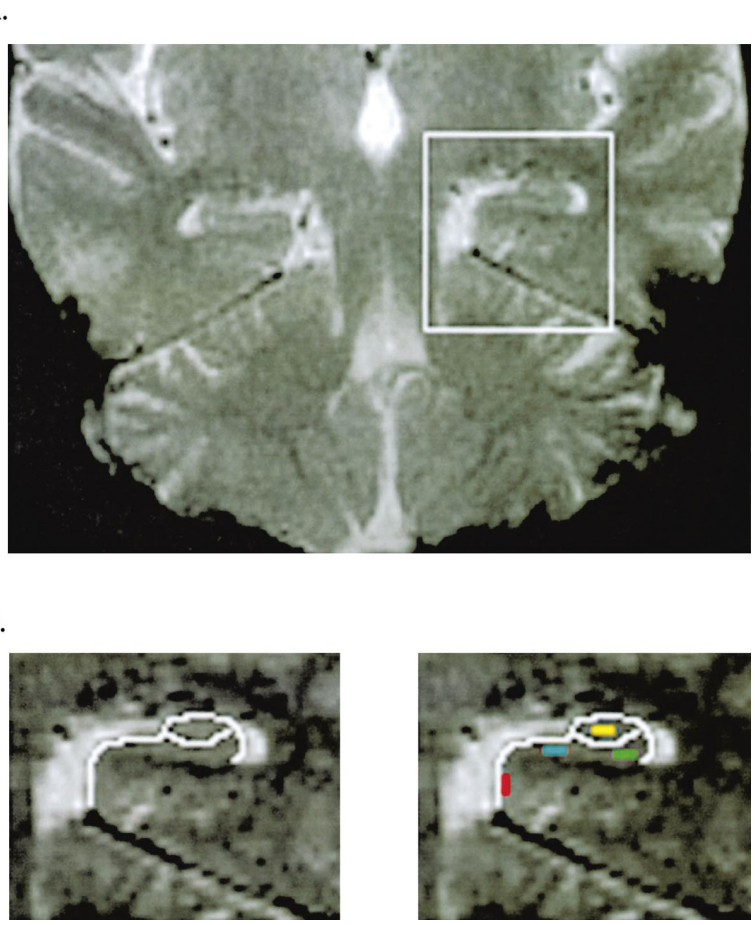

Figure 1. Resting T2*-Weighted Images Provide the Needed Resolution to Visualize the Subregions of the Hippocampus in Humans

(A) One of five transverse slices through the hippocampal formation. This slice was selected because it provided optimal visualization of the anatomical landmarks and because it was anterior to the lateral geniculate nucleus.

(B) Magnified view of the hippocampus. Left panel: the external morphology as well as the internal white matter tracts of the hippocampus are identified (white lines). Right panel: pixels within the four hippocampal subregions are sampled for analysis (red = entorhinal cortex, blue = subiculum, green = CA1, yellow = dentate gyrus/CA3). Borderzones between the subregions are purposefully avoided. The dentate gyrus and the CA3 subfield are combined.

vessels as well in brain (Ogawa et al., 1990) and cardiac tissue (Atalay et al., 1993), we reasoned that a methodology based on resting T2* signal could map neuronal function and do so with improved spatial resolution. Furthermore, because measurement of resting signal would not require an activation task, the MRI approach would become applicable to mapping dysfunction in both human subjects and mice.

To test this idea, we explored whether resting gradient-echo T2*-weighted images could detect alterations in resting neuronal function. Earlier studies in brain and cardiac tissue (Ogawa et al., 1990; Atalay et al., 1993) have shown that resting gradient-echo T2*-weighted signal varies with resting oxygen (ROXY) levels. We have developed an MRI protocol based of these findings that yields high-resolution images of the individual hippocampal subregions and have used this method to evaluate differences in signal intensity of the hippocampal subregions in both aging humans and in genetically modified mice. Among healthy humans with normal cognition, signal from the subiculum, the output region of the hippocampus, provided the best indicator of the performance of subjects on hippocampal-dependent memory tasks. Indeed, we found a correlation between the intensity of the signal from the subiculum and delayed memory recall. We then turned to examine two populations of healthy elderly people, those with and those without hippocampal-dependent memory decline, and detected in subjects with a decline in memory a reduction in the overall signal from the hippocampus compared to normal age matched subjects.

To determine whether changes in magnitude of the signal found in subjects with memory decline can be produced by functional alterations of the brain without cell or fiber loss, we turned to genetically modified mice with which we could carry out detailed anatomical analyses. For this purpose, we used R(AB) transgenic mice (Abel et al., 1997) that express in the hippocampus and forebrain a dominant-negative inhibitor of protein kinase A (PKA). The transgene causes a change in the "resting" biochemistry of neurons by reducing basal PKA activity by 50%. Neurons that express this transgene have deficits in long-term potentiation (LTP), a cellular mechanism of plasticity underlying memory, and, in fact, mice expressing the transgene phenocopy age-related memory decline with deficits in hippocampal-dependent memory function, including an instability in their internal representation of space (Rotenberg et al., 2000). These mice show a reduction in the intensity of ROXY-dependent signal in the hippocampus compared to wild-type littermates, and this reduction is comparable quantitatively to that of the age-related memory decline in humans. Since in these animals we find no loss of cells or fiber tracts based on a variety of molecular markers, the reduction in signal in these animals is attributable to a physiologic defect and not the consequence of anatomi-

cal changes in the hippocampus. Thus, this MRI method is sufficiently restricted spatially to detect subregional changes, and it is sufficiently sensitive functionally to detect reductions in signals that reflect alterations in hippocampal function that are significant for cognitive performance yet do not reflect detectable anatomical loss.

Results

Applying a High-Resolution MRI Method for Generating Hippocampal Brain Maps in Humans that Are Heavily T2* Weighted and Preserve Anatomical Resolution

We carried out all of our human scanning studies using a GE 1.5 tesla magnet. In a series of preliminary studies, we determined the parameters of a pulse sequence that gave us optimal resolution of the hippocampal formation. The T2* (magnetic susceptibility) effect was optimized by titrating flip angle and echo time to the point where the lateral temporal lobe was obscured by susceptibility artifact caused by the petrous bone (see Figure 1). We then improved the signal to noise by increasing number of excitations, and we minimized the scan duration by manipulating repeat time (TR) and matrix size.

An example of a brain image acquired with the final pulse sequence is shown in Figure 1. Acquisition time was 3 hr and 54 min. The susceptibility artifact extending into the lateral but not medial temporal lobes demonstrates the heavy T2* weighting. In contrast to dynamic T2* images, the resting T2* image provided the anatomical resolution necessary to identify not only the external morphology of the hippocampal formation, but also its internal white matter tracts. Using these anatomical landmarks (Figure 1), one can identify the four critical subregions of the hippocampal formation: (1) the entorhinal cortex, (2) the dentate gyrus/CA3 subregion, (3) the CA1 subregion, and (4) the subiculum. Because we could not visualize reliable landmarks that distinguish the dentate gyrus from the CA3 subfield, these two regions were combined. Pixels for analysis were chosen from within these general regions, thus averting the concern over defining the precise borderzones between subregions, which requires histological staining (Amaral and Insausti, 1990).

Hippocampal Signal in the Subiculum Is Selectively Correlated with Normal Memory Recall in Healthy Elderly Subjects

We examined a total of 30 subjects selected from a prospective community-based study on aging. All these subjects had been followed for at least 5 years with detailed annual medical, neurological, and neuropsychological evaluations and were found to be healthy, without dementia or other neurological or psychiatric disorders. We evaluated their neuropsychological test scores and determined that 14 subjects had stable and normal memory performance over time, while 16 subjects had a decline in memory performance over time (Small et al., 1999). The mean age of both normal memory groups was 78 years, and the mean age of the memory decline group was 79 years. Eighty-five percent of the memory decline group and 78% of the stable memory group were women.

We focused first on the 14 normal subjects with normal memory. This group performed normally on all tests at every annual evaluation and showed no evidence of cognitive decline over time. It therefore is unlikely that any of these subjects had early Alzheimer's disease (Mayeux and Small, 2000). We therefore asked: Does the variance in signal from any region of the hippocampal formation of this normal group of elderly subjects correlate with variance in performance in any cognitive domain? To address this question, we carried out a multivariate linear regression and found that only signal from the subiculum, and not from any other region in the hippocampus, was significantly correlated with the delayed recall component of a word-list memory task, the Selective Reminding Test (Buschke and Fuld, 1974) ($R = 0.67$; $F = 9.6$; $p < 0.005$). This correlation was selective for memory performance; it was not found between signal and performance scores on other cognitive measures of abstract reasoning, language, or visuospatial ability (Figure 2). Thus, the ability to evaluate each of the subregions of the hippocampus simultaneously has allowed us to pinpoint the subiculum as being most directly associated with a delayed recall memory task. This opens up the possibility of determining whether other facets of memory, such as encoding, consolidation, or recall, or other memory paradigms are differentially associated with different subregions.

Brain Maps Based on Resting T2* Detect a Reduced Hippocampal Signal among Human Subjects with Memory Decline

We next turned to a comparison of the group of elderly subjects with normal memory with the age-matched group of subjects with memory decline. We carried out a multivariate analysis and found that, as a group, the subjects with memory decline had significantly lower intensity signal from the hippocampus compared to age-matched controls ($F = 3.1$, $p < 0.05$). By contrast, no difference was found between the groups in average signal intensity from the dentate nucleus of the cerebellum ($t = 0.84$; $p = 0.4$). As shown in Figure 3B, univariate analysis revealed that among all hippocampal subregions, only the entorhinal cortex was significantly different between the groups ($F = 7$, $p < 0.05$). The entorhinal cortex is the first subregion of the hippocampus targeted by AD pathology (Braak and Braak, 1996), and the entorhinal cortex has been the focus of structural as well as functional MRI studies (Bobinski et al., 1999; Small et al., 1999) in an effort to detect the earliest stage of the disease. It will be of interest to follow this population to determine whether the subjects with diminished signal from the entorhinal cortex are at greater risk to progress to Alzheimer's dementia.

Indeed, a selective reduction in signal from the entorhinal cortex was not the only pattern among the subjects. Figure 3B shows the pattern of signal across all hippocampal subregions standardized to the normal group. This finding raises the interesting possibility that, in principle, ROXY imaging might be used to fractionate age-related memory loss into a number of regionally and perhaps etiologically distinct categories (Small, 2000).

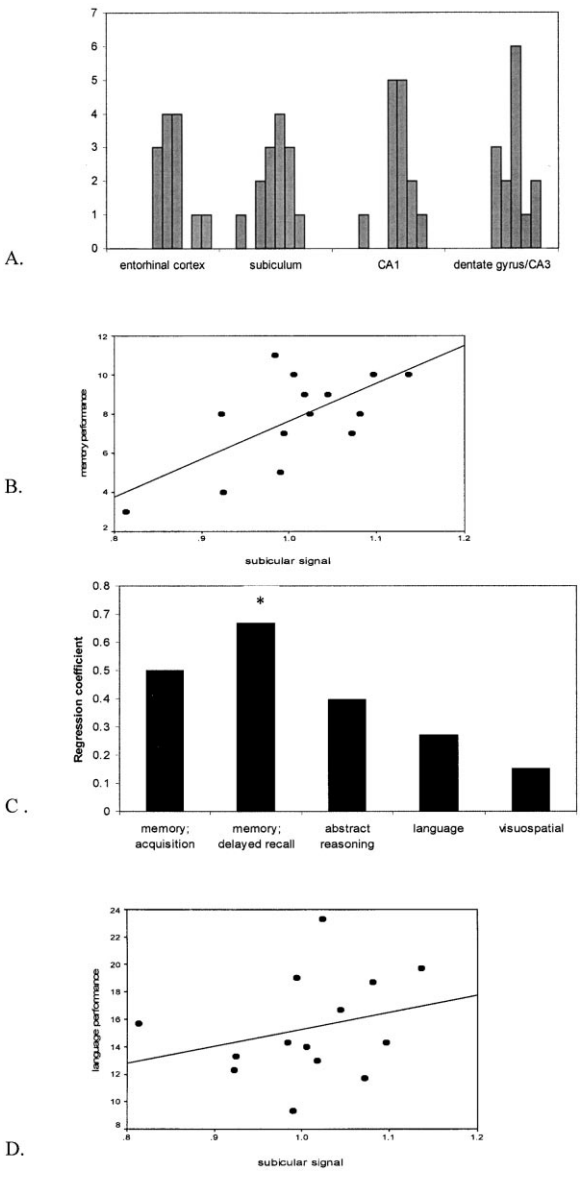

Figure 2. Hippocampal Signal in the Subiculum Is Correlated Selectively with Normal Memory Recall in Healthy Elderly Subjects

(A) The distribution of signal of the hippocampal subregions among the healthy subjects.

(B) There is a significant correlation between the subicular signal and performance on the delayed recall component of a word-list memory task—the SRT (Buschke and Fuld, 1974). No correlation was found between this measure and signal from other hippocampal subregions.

(C) The correlation between subicular signal and memory performance was selective (asterisk). No correlation was found between hippocampal signal and other cognitive tasks such as abstract reasoning (WAIS similarities; Wechsler, 1981), language (CFL; Benton, 1967), or visuospatial ability (The Rosen Drawing Test; Rosen, 1981).

(D) Subicular signal and performance on a measure of language function are not correlated.

In R(AB) Transgenic Mice Brain Maps Detect Reduced CA1 Hippocampal Signal Compared to Wild-Type Mice

Although the observed correlation among healthy elders suggests that MRI method can measure physiological function, we cannot, based on these observations, know whether the signal is sufficiently sensitive to reflect functional changes that occur even in the absence of anatomical damage. We therefore turned to parallel studies in genetically modified mice where one can correlate functional disturbances with anatomical ones.

To study the hippocampal signal in rodents who had a memory defect comparable to that we studied in humans, we used the R(AB) genetically modified mice. These mice express a dominant-negative inhibitor of the cAMP-dependent protein kinase (PKA) that is targeted specifically to the forebrain and hippocampus. The transgene causes deficits in resting molecular function with a 50% reduction in basal PKA activity. As is the case with elderly mice (Bach et al., 1999), the R(AB) transgenic mice have a selective defect for the late phase of LTP and a selective defect for long- but not short-term memory in hippocampal-based spatial and nonspatial tasks (Abel et al., 1997; Bach et al., 1999).

In preliminary experiments, we again determined the parameters of a pulse sequence that provided an optimal map of the mouse brain, with particular emphasis on the hippocampal formation; we carried out MRI scanning with a 4.23 tesla magnet (Wang NMR, Livermore, CA; console by High-Field NMR Systems, Birmingham, AL), with a head radio-frequency coil specifically designed for imaging the mouse brain. The final pulse sequence again provided a brain map that is heavily T2* weighted (Figure 3). At the same time, the map provides sufficient anatomical resolution to identify the external morphology of the hippocampal formation and its internal white matter tracts. These anatomical landmarks allow the CA1 subregion to be identified (Figure 4). With this protocol, we scanned 20 mice—10 R(AB) mutants and 10 wild-type littermates. All mice were anesthetized during scanning and monitored with pulse oximetry. Scans were acquired and analyzed blindly.

We then averaged signal intensity from the CA1 subregion of each mouse to determine whether the resting T2* signal could detect physiologic deficits in the CA1 region. To control for interindividual variability in global signal intensity, we used average signal intensity from the subcortex—i.e., brain areas that do not express the transgene—of each mouse to normalize the CA1 signal intensity. Examples of these images in wild-type mice are shown in Figures 5A and 5B. As evident in Figure 5C, the normalized signal intensity of the mutant mice ($n = 10$, average $= 1.1$, SD $= 0.08$) was significantly lower than the normalized signal intensity of the wild-type littermates ($n = 10$, average $= 1.2$, SD $= 0.1$) ($t = 2.6$, $p < 0.05$).

These studies therefore reveal two interesting findings. One, they show that the genetic defect that phenocopies the age-related memory deficits also phenocopies the human age-related defect in hippocampal signal. Second, in contrast to the current finding, where the hippocampus was assessed in an intact brain of an alive animal, the basal PKA deficit caused by the transgene was not revealed by the resting electrophysiological profile of neurons that were investigated in an in vitro hippocampal slice preparation (Abel et al., 1997). These findings, therefore, highlight an advantage of assessing brain physiology in vivo.

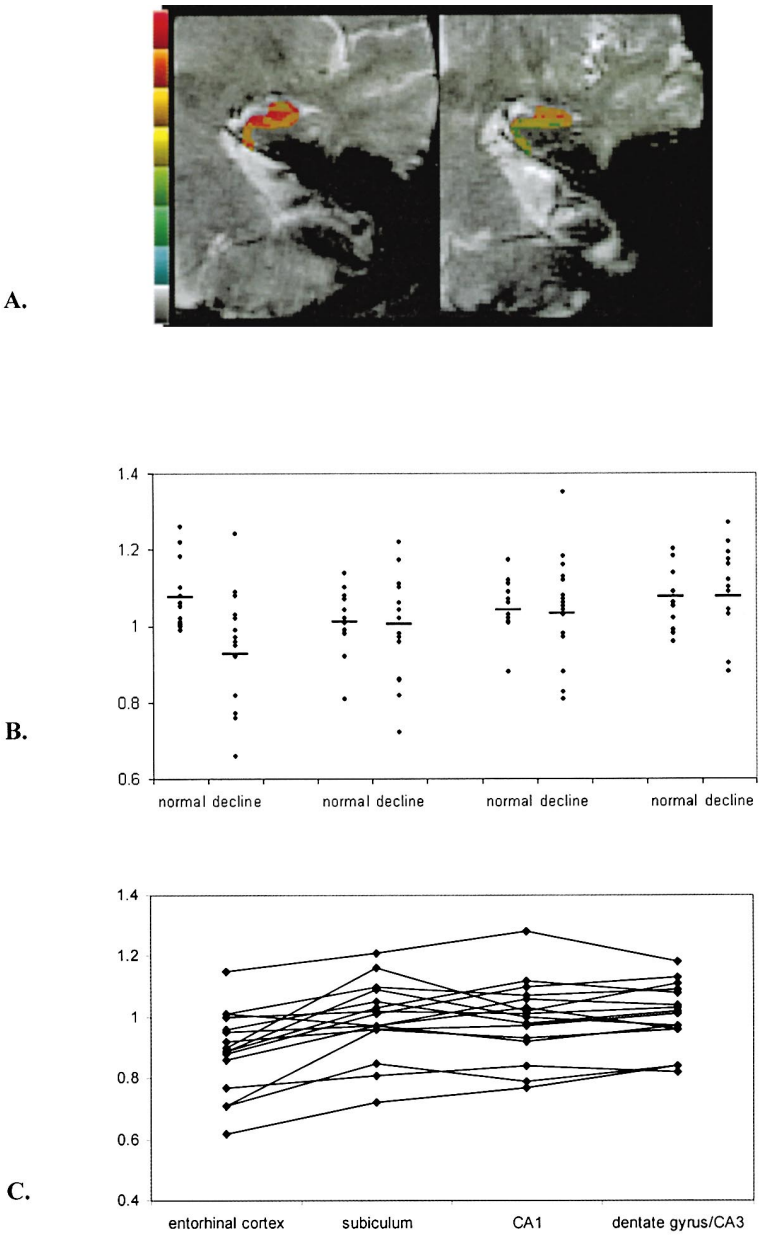

Figure 3. Maps Based on Resting T2* Can Detect a Reduced Hippocampal Signal from Human Subjects with Memory Decline

(A) An example of hippocampal signal intensity in an elderly subject with normal memory (left) and an age-matched subject with memory decline (right). Signal intensity is normalized to global signal and color coded; blue reflects lowest and red highest signal intensity.

(B) Average signal intensity for each hippocampal subregion was lower among the 16 subjects with memory decline compared to the 14 subjects with normal memory. A significant effect was found for the hippocampus globally, with the greatest trend in reduced signal found in the entorhinal cortex. Error bars represent standard error of the mean.

(C) Analyzing the hippocampal subregions as a circuit can reveal individual patterns of hippocampal dysfunction. Patterns may reflect different etiologies of memory decline.

Reduced CA1 Signal in Transgenic Mice Is Not Caused by Structural Lesions

Does a reduced signal in the CA1 region of the hippocampus necessarily imply a structural lesion? Prior studies have established that there is diminished basal PKA activity in the CA1 subregion in the R(AB) mutants compared to wild-type littermates. Detailed histological analyses, however, were not performed in these studies. The possibility remained, therefore, that the reduced CA1 signal detected by the brain maps were associated with primarily structural and not necessarily physiologic lesions. To address this issue, we first carried out cell counts in the dentate gyrus and the CA1 and CA3 regions and found no differences in numbers of cell bodies in wild-type and mutant mice (Figure 6). We next performed systematic histochemical analysis of both transgenic and wild-type mice (Figure 7). Four R(AB) transgenic and three wild-type mice, selected at random from the MRI scanned groups, were perfused with paraformaldehyde and their vibratome-sectioned brains were analyzed using Nissl stain to detect cellular loss and Timm stain to detect the pathways within the hippocampus. We again found no difference between wild types and R(AB). In addition, we have used four molecular markers: (1) MAP2, the major microtubule-associated protein in neurons that stains for dendrites; (2) GAP-43, a neural-specific growth-associated protein and a marker of axonal growth and presynaptic terminals that stains in particular the perforant pathway input to both the dentate gyrus and the CA3 and CA1 regions; (3) synaptophysin, a major synaptic vesicle protein that is a marker for presynaptic terminals and stains the mossy fiber and

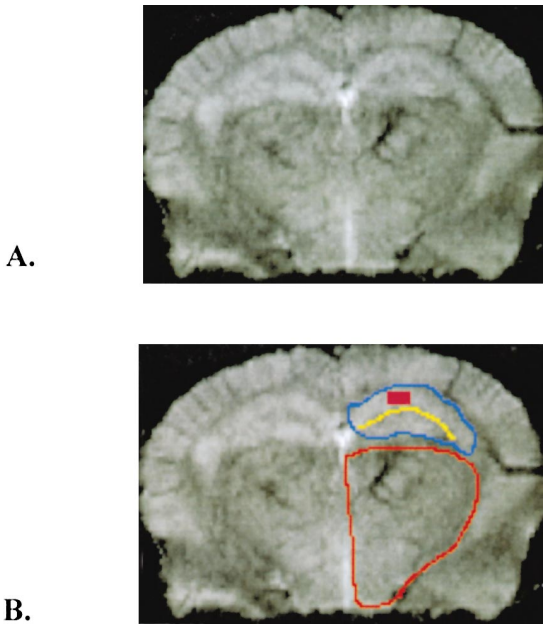

Figure 4. Resting T2*-Weighted Images Provide the Resolution Needed to Visualize the CA1 Subregion in Mice

(A) A coronal slice acquired through the dorsal hippocampal formation.

(B) The external morphology of the hippocampus is identified (blue line). The internal white matter tracts are identified (yellow line). A region in the CA1 subfield is selected (red square). A region in the subcortex is selected (red line) that is used to normalize the CA1 signal intensity.

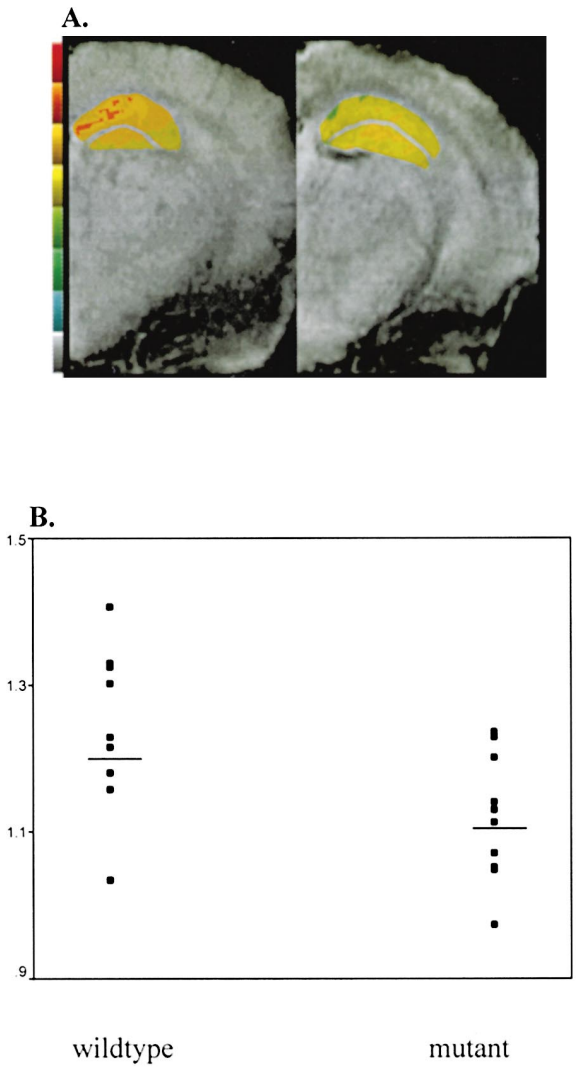

Figure 5. In R(AB) Transgenic Mice Brain Maps Detect Reduced CA1 Hippocampal Signal Compared to Wild-Type Mice

(A) An example of hippocampal signal intensity in an a wild-type mouse (left) and a littermate mutant expression, the R(AB) transgene (right). Signal intensity is normalized to global signal and color coded. Average signal intensity in the CA1 subregion was significantly lower among the 10 mutant mice compared to 10 wild-type littermates. Error bars represent standard error of the mean.

Schaffer collateral projections; and (4) Calbindin-D, a Ca^{2+} binding protein and a marker for many types of interneurons. In the hippocampus, it is expressed in all dentate granule cells. All tissue samples were analyzed blindly for these markers, and no significant difference was observed between the transgenic and wild-type littermates (Figure 7). Finally, because iron can influence T2* signal (Ogg et al., 1999), we performed a Perls staining protocol (Hill and Switzer, 1984) for non-heme iron localization in the brain and found no difference in the hippocampus of mutant and wild-type animals (Figure 7G). These studies clearly demonstrate that a reduced ROXY-dependent signal can be detected in the absence of structural change. Thus, as indicated above, the imaged signal is more sensitive than either structural or electrophysiological methods of detection.

Demonstrating that the MRI Method Is Related to T2* and that the Signal Is Influenced by Oxygen Concentrations

The pulse sequence of the MRI protocol was designed to be heavily T2* weighted. Nevertheless, we cannot rule out the possibility that the differences in signal observed in the above studies were not caused, or at least influenced, by other components of the signal. In a landmark study, Ogawa and colleagues used a similar T2*-weighted gradient-echo MRI protocol to show signal differences in the white and gray matter of the rodent hippocampus (Ogawa et al., 1990). To demonstrate that the effect was in fact related to T2* signal, they showed that signal contrast increased with increasing echo time. We therefore reimaged two subjects from the normal memory group who on initial imaging were found to have differences in signal from the subiculum.

We imaged the hippocampus of these subjects with the same gradient-echo pulse sequence as described above but at various echo times. The original and follow-up images acquired at a TE of 45 ms are shown in Figure 8. The time interval between scanning was 2–3 months showing that the method produces relatively stable images over time. Furthermore, in order to assess rest-retest reliability, we determined the normalized signal from the subiculum in the follow-up images and found

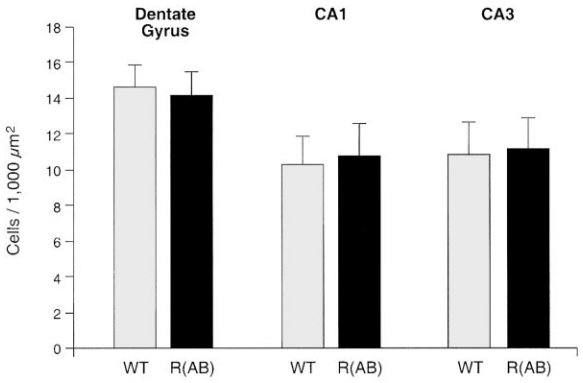

Figure 6. Reduced CA1 Signal in Transgenic Mice Is Not Caused by Cell Loss

R(AB) mice and wild-type mice have similar cell densities. Cell counts: neuronal cell densities in the hippocampi of transgenic R(AB) mice and wild-type (wt) littermates were counted on flouro-Nissl-stained vibratome sections. The average neuronal densities with the standard deviations are indicated (cell number/100 μm^2) and do not significantly differ between wild-type (white bars) and mutant (black bars) animals.

that both subjects maintained the same rank ordering established with the baseline images.

In order to assess whether the contrast in signal between the subjects was related to T2*, we explored the relationship between echo time and signal contrast. As shown in Figure 8, the difference in subicular signal increased with increasing echo time. A spin-echo pulse sequence was also (TR = 300 ms; TE = 60 ms) used to acquire signal from the subiculum, which, as shown in Figure 8, eliminated the between subject difference. These findings suggest the signal is related to T2*, although, because the graph in Figure 8C does not extrapolate to zero, there are other sources of signal that contribute to the between-brain contrast.

We also tested whether we could influence hippocampal signal by systematically manipulating the concentration of oxygen breathed by a mouse during scanning. In a series of scanning periods, we recorded hippocampal signal from a wild-type mouse that breathed room air, 100% oxygen, and then room air again. Hippocampal signal was measured in these three consecutive conditions and, as shown in Figure 8, CA1 signal changed in parallel to oxygen concentration.

Discussion

Imaging the Function of Hippocampal Subregions in Mice and Men

We have developed a novel application of high-resolution ROXY-dependent MRI that allows the function of multiple hippocampal subregions to be interrogated in both human subjects and mice. Earlier studies have used measurements of resting T2*-weighted signal to detect external manipulations of resting blood oxygenation in brain and cardiac tissue (Ogawa et al., 1990) or to map primary motor cortex using covariance patterns of resting signal (Biswal et al., 1995; Xiong et al., 1999). A recent study by Teicher et al. (2000) showed that resting T2 relaxation time in the basal ganglia was correlated with behavioral measures in children with attention deficit/hyperactivity disorder; however, the neuronal correlate of the T2 relaxation time signal is not known since the method has not yet been explored in animals. Our study represents an attempt to use MRI methodology to evaluate neuronal function of multiple hippocampal subregions in parallel human and mice studies.

Imaging Resting T2* Signal Yields Increased Spatial Resolution

Compared to other functional imaging modalities that assess "resting" physiology—such as positron emission tomography, single-photon electron computerized tomography, near-infrared spectroscopy, and magnetic resonance spectroscopy—a main advantage provided by MRI is superior spatial resolution. Although fMRI has been shown to evaluate the hippocampal formation globally, echo-planar fMRI imaging has a resolution of within the single millimeter range. Despite limited success (Gabrieli et al., 1997; Small et al., 1999), analysis of the hippocampal subregions is best accomplished with a submillimeter resolution, factoring in spatial error introduced by image acquisition and processing. One of the advantages of mapping ROXY-dependent signal is that the resolution of the brain maps is enhanced. We here demonstrate that we can localize subregions of the hippocampus in the submillimeter range.

In most dynamic fMRI studies, a high-resolution "anatomic" image is acquired separately from the "functional" image, and the two images are coregistered. The spatial error introduced by coregistration programs specifically developed for this purpose is acceptably low when imaging large brain volumes. However, for smaller brain regions such as the hippocampal subregions, slight misregistration can significantly effect the validity of analysis. Here, we were able to generate brain maps that highlighted the external and internal architecture of the hippocampal formation. Thus, anatomical landmarks required to identify the hippocampal subregions were identified in the primary image, obviating the need for coregistration onto a second image and precluding a potential source of error.

The importance of this enhanced resolution is illustrated in evaluating hippocampal dysfunction. The cells of the hippocampal subregions have unique patterns of gene expression that are reflected in the cells' biophysical properties and receptor profiles. These differences account for the fact that cells of the different subregions are selectively vulnerable to different pathological processes (Small, 2000). Thus, evaluating the hippocampus globally does not do full justice to its cellular complexity. Precise mapping of dysfunction requires a subregional analysis. Similar arguments extend to the related utility of ROXY-dependent imaging as a functional assay for neuropharmacological intervention.

Imaging Hippocampal Subregions of Elderly Subjects with Normal Memory and with Memory Decline

We first examined subjects with normal memory who had been followed for at least 5 years and underwent detailed annual medical, neurological, and neuropsy-

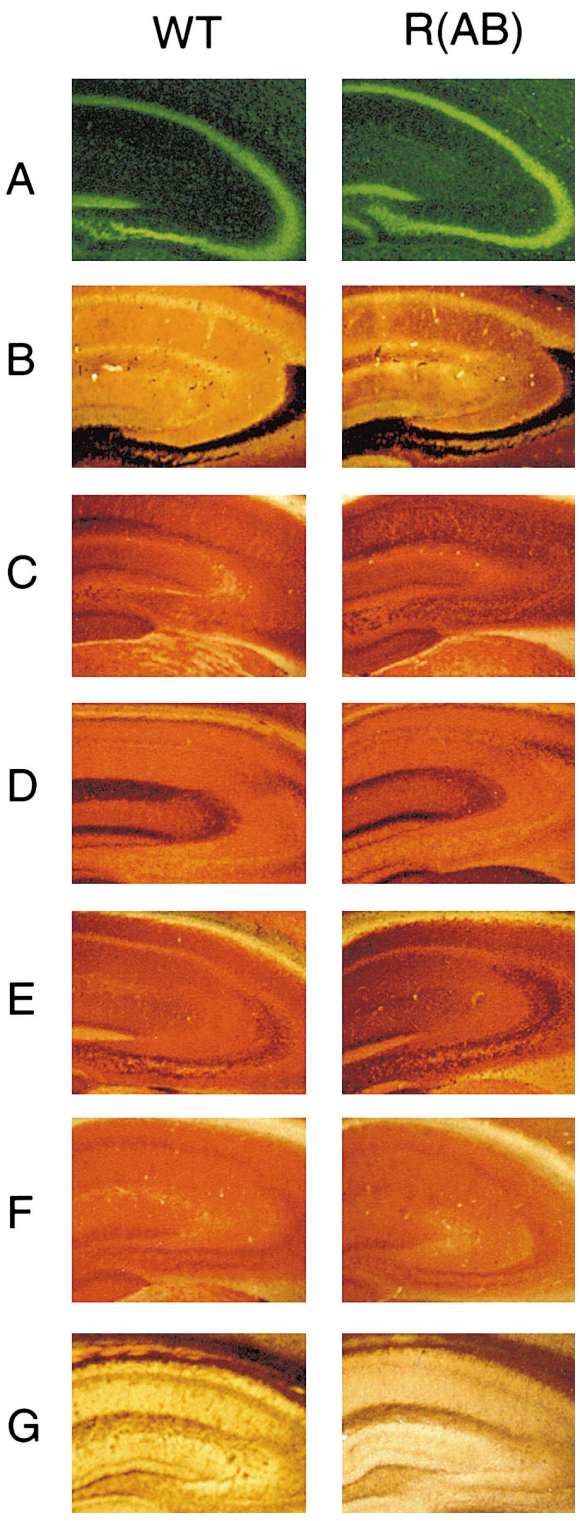

Figure 7. Reduced CA1 Signal in Transgenic Mice Is Not Caused by Structural Lesions

Immunohistochemical analysis of the hippocampus of wild-type (wt) and R(AB) transgenic mice. Floating vibratome sections (50 μM) location matched between control and mutant animal were stained with fluoro-Nissl stain (A), Timm stain (B), and with antibodies against MAP2 (C), GAP43 (D), synaptophysin (E), calbindin (F), and non-heme iron, indicated by the dark staining by Perls solution (G). No significant differences in hippocampal cytoarchitecture were found between the R(AB) and wild-type littermates using these probes. In addition, no differences were observed outside of the hippocampal area using the above histochemical probes and acetylcholinesterase staining (data not shown).

chological evaluations. At every annual evaluation, the subjects in this group performed normally on all tests. Moreover, longitudinal analysis showed that the subjects' memory performance did not decline over time. Taken together, this cognitive profile makes it exceedingly unlikely that these subjects have abnormalities of hippocampal function that can be caused by early Alzheimer's disease. In these 14 subjects, the strength of the signal from the subiculum was linearly correlated with performance on components of the Selective Reminding Test (Buschke and Fuld, 1974) but not with performance in other nonmemory-related cognitive tests—abstract reasoning, language, and visuospatial ability. The specific correlation with memory performance provides evidence for the possibility that different subregions of the hippocampal formation may prove to have different functions in different memory-related tasks. These results also provide an independent validation of this methodology by showing that the signal generated from the hippocampal formation reflects a cognitively relevant physiological function.

In addition to detecting differences among normal subjects, ROXY-dependent imaging distinguished elderly subjects with and without hippocampal-based memory decline. The subjects with memory decline had a decline in T2* signal from the hippocampus. Age-related memory decline among otherwise healthy nondemented elderly is etiologically heterogeneous. Since early preclinical Alzheimer's disease is one contributing cause to age-related memory decline and some subjects in the memory decline group might well have preclinical Alzheimer's disease, these subjects may, in fact, have cell loss in the entorhinal cortex (Gomez-Isla et al., 1996) that could account for the diminished signal intensity in this subregion. However, most causes of age-related memory decline, including Alzheimer's pathology, result in physiologic dysfunction before cell loss. It therefore was important to establish whether the MRI method that we have here developed could detect pure physiologic deficits.

ROXY-Dependent Imaging Is Sufficiently Sensitive to Detect Functional Changes in the Absence of Anatomical Changes

The importance of determining whether the MRI method can detect physiological deficits without detectable neuronal loss illustrates why it is helpful to be able to carry out parallel animal studies. We have developed the MRI methodology to be applicable to mice because of the unique experimental advantage afforded by investigating mice with genetic lesions. We used R(AB) transgenic mice because they have hippocampal-based deficits that behaviorally phenocopies age-related memory decline. The transgene causes a 50% reduction in resting PKA, which is reflected by impaired LTP in the CA1 region. We found that ROXY-dependent imaging was able to map this molecular lesion and to detect a

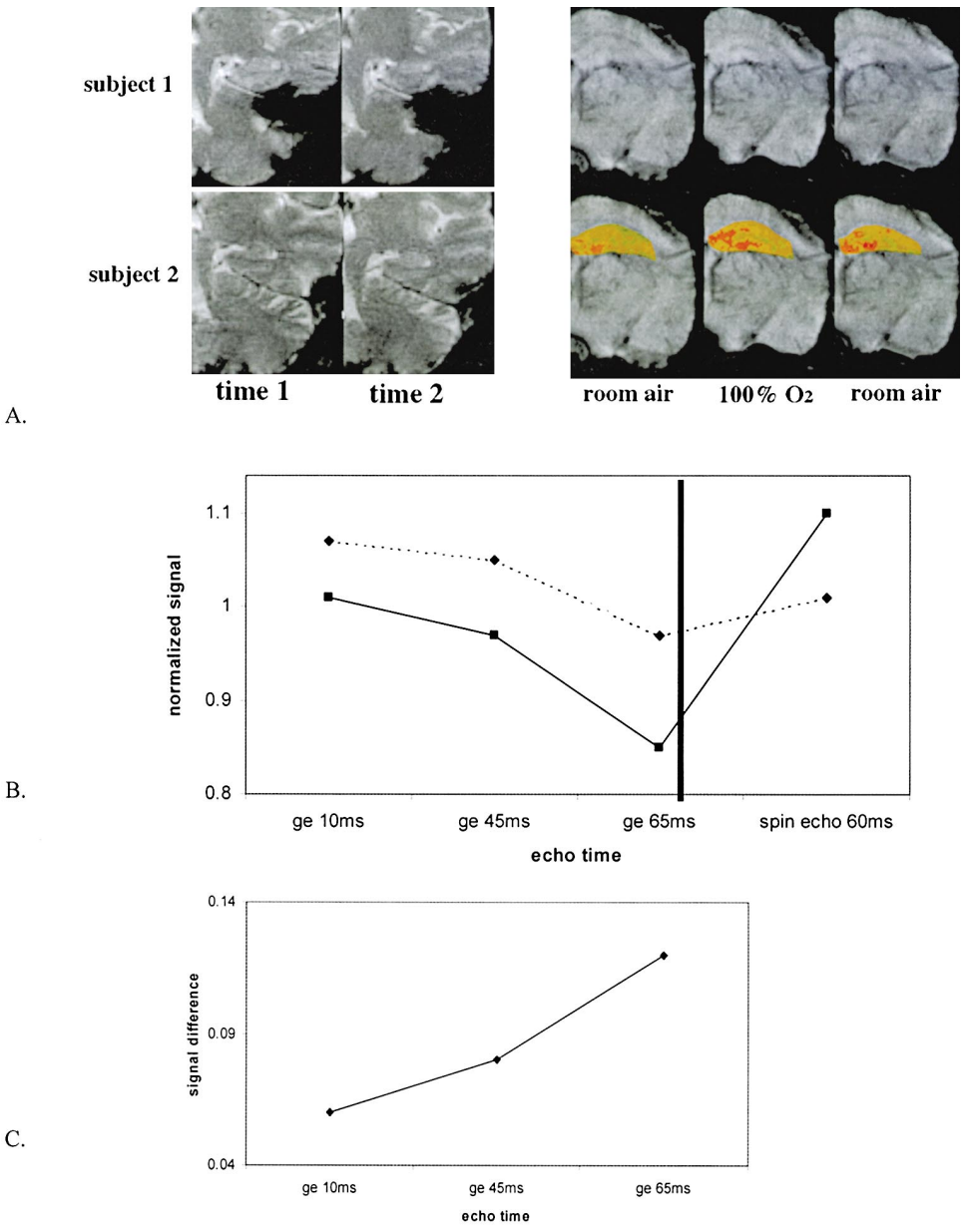

Figure 8. Hippocampal Signal Is Related to T2* and Is Influenced by Oxygen Concentration

(A) Left panel: repeat scanning was performed on two subjects with baseline differences in subicular signal. The baseline and follow-up images were acquired 2–3 months apart. Right panel: a wild-type mouse was scanned consecutively while on room air, 100% oxygen, and on again on room air. Hippocampal signal changed in parallel to changes in oxygen concentrations.

(B) Normalized gradient-echo signal from the subiculum was measured with varying echo-times. Each subject was plotted separately. Signal measured with a spin-echo pulse sequence eliminated the difference between the subjects.

(C) A plot of the difference between the two subjects at each echo time shows that the difference increases with increasing echo time.

reduced signal in the CA1 region of the transgenic mice compared to wild-type littermates. By carrying out cell counts and other histological analyses, we could document that the reduced signal did not result from obvious structural changes in CA1 neurons. The detected difference in signal could result from deficits in LTP since mechanisms of cellular plasticity can influence the resting firing patterns of hippocampal neurons as measured in vivo in intact brains (King et al., 1999).

These findings constitute a demonstration that an MRI technique can detect a pure physiologic lesion. This is important because many causes of brain dysfunction are likely to cause physiological deficits without changes in neuronal structure. This is true for many causes of hippocampal dysfunction and is even true for the earliest stages of Alzheimer's disease, where physiological deficits antedate cell loss. These physiological lesions cannot be detected microscopically by analyzing brain tissue accessed by biopsy or at autopsy. Together with its anatomical resolution, ROXY-dependent imaging

provides a physiological biopsy of the hippocampal subregions.

ROXY Imaging Has a Variety of Other Applications

ROXY-dependent imaging is likely to be useful in addressing a variety of questions. First, by not relying on subject participation, this method will be useful for mapping diseases of the brain in which it is impractical to obtain patient participation for performing an activation task. This is true for all brain disorders that affect infants or young children, for many brain disorders that influence cognition such as mental retardation and dementia, and for investigating brain disorders that require anesthesia for imaging, such as some psychiatric illnesses and movement disorders. Second, even in patients who can engage in an activation task, the enhanced spatial resolution provided by ROXY-dependent imaging makes it possible to map disease more precisely. Third, the high resolution of ROXY-dependent imaging makes it suitable for studies of regional restriction of gene expression in mice, as we illustrate here. Fourth, since ROXY-dependent imaging allows one to interrogate many brain regions simultaneously, it should be useful in mice for establishing the functional locus of a genetic modification when the anatomical consequences of that modification are not clearly known. Finally, this methodology opens up the possibility to investigate, in parallel, the human nervous system and that of genetically modified mice for the study of both normal function and in models of disease using, in each case, homologous measures of brain function.

Limitations to ROXY-Dependent Imaging

Despite these advantages, there are also important limitations to this methodology. By looking only at basal function, one may fail to detect abnormal function that might emerge with the increase in sensitivity brought out by a behavioral task. Thus, as with other measures of tissue function such as the EKG or the EEG, measurements of resting function may fail to detect deficits that are revealed better by actively testing or stressing an organ system. When subjects can perform a task, it may therefore be preferable, for many purposes, to use dynamic fMRI.

In addition, there are a number of potential sources of error that may diminish the specificity of T2* signal for detecting neuronal dysfunction. One source is based on factors that alter signal intensity independent of the concentrations of resting oxyhemoglobin. In the current approach, both T1 and proton density effects contributed to signal intensity. Although we are currently developing an approach that will provide a purer T2* map, disease states or experimental manipulations may result in alterations—such as gliosis or edema—that would modify T2* signal independent of oxyhemoglobin/deoxyhemoglobin ratios. Iron is a particular concern since its brain concentration increases with age and with disease (Loeffler et al., 1995), and iron has a clear effect on T2* signal (Ogg et al., 1999). Although the hippocampal formation has a relatively low concentration of iron, with the highest concentrations found in the basal ganglia (Hallgren and Sourander, 1958), we nonetheless carried out a Perls staining protocol for non-heme iron and failed to detect a difference in iron concentration in the hippocampus of mutant and wild-type animals, thus providing assurance that the observed differences in the signal are not due to differences in concentration or localization of iron. A second source of diminished specificity are factors that alter resting oxyhemoglobin concentrations independent of neuronal metabolism. Although global signal intensity was used to normalize against sources of global signal variability, this does not address potential sources of local variability. For example, if a brain disease or an experimental manipulation were to modify the integrity of blood vessels and do so with subregion selectivity, this would influence T2* signal independent of neuronal dysfunction and result in false positive findings.

Many of these potential sources of error, which are true for dynamic task-induced as well as resting fMRI studies, are best addressed by coupling the MRI technique with other modes of investigation that can directly rule out alternative sources of signal change. It is for these reasons that having available an identical MRI approach suitable for mice as well as humans is particularly valuable.

Experimental Procedures

Humans

Subjects

Subjects were selected from a prospective study of individuals 65 years and older who reside in a single community in Northern Manhattan and who receive annual medical, neurological, and neuropsychological exams. All subjects were presented in a consensus conference comprised of neurologists, psychiatrists, and neuropsychologists and were excluded if at any time point they fulfilled DSM-IV criteria for dementia or if they were diagnosed with stroke, Parkinson's disease, or depression. Subjects were also excluded if they were diagnosed with "questionable dementia," a category applied if neuropsychological test performance was below cutoff scores established for this community (Stern et al., 1992) but were not extensive enough to fulfill dementia criteria. A slope of memory performance over time was calculated for each subject by performing a linear regression of the total recall score of the Selective Reminding Test (Buschke and Fuld, 1974), a measure of declarative memory. Subjects were assigned to the memory decline group if their memory performance worsened with time and were assigned to the normal memory group if their memory did not decline over time.

Image Acquisition and Processing

A sagittal image was acquired to identify the long axis of the hippocampal formation. Five 5 mm oblique coronal slices were selected perpendicular to long axis of the hippocampal formation. The most anterior slice was always placed at the posterior aspect of the uncus, thus assuring the imaging of a slice appropriate for subregion localization.

Foam pads and surgical tape were used to secure the head. Subjects were instructed to close their eyes and scanning was performed using a 1.5 tesla scanner. Gradient-echo T2*-weighted images were acquired (TE/TR = 45/300; flip angle = 10; NEX = 3; in-plane resolution = 0.86 mm × 0.86 mm.

Postacquisition data processing was performed on a Silicon Graphics Indigo II work station, using image display and analysis software packages (IDL Research Systems). Data analysis was performed on a PC-based Linux workstation using MEDx (Sensor Systems) and was performed by an investigator blinded to subject grouping.

A single slice that provided optimal visualization of the required anatomical landmarks was selected. Importantly, this slice was never more than 20 mm posterior to the amygdala and was always anterior to the lateral geniculate body, assuring that the slice con-

tained the entorhinal cortex (Amaral and Insausti, 1990). As illustrated in Figure 1, the external morphology of the hippocampus and the white matter tracts within the hippocampus were identified. These landmarks were used to identify the general locale of the hippocampal subregions.

Data Analysis

The average signal intensity of the pixels selected within each hippocampal subregion, for each subject, was determined. The average signal intensity of the dentate nucleus of the cerebellum was also determined. To control for interindividual variance in signals not related to hippocampal function, the average signal intensity of each subregion was divided by this global signal measured from the slice, yielding normalized signal intensities.

The independent variable of the MANOVA was group (memory decline versus controls) and the dependent variables were normalized signal intensity acquired in the entorhinal cortex, the subiculum, the CA1 subfield, and the combined dentate gyrus/CA3 subregion. Age and gender were included in the extended model as covariates.

The independent variables used in the linear regression were the normalized signal intensity from the four hippocampal subregions, and the dependent variables were performance scores on measures of memory (total recall and delayed recall component of the Selective Reminding Test [Buschke and Fuld, 1974]), abstract reasoning (the WAIS-R similarities [Wechsler, 1981]), language (the Controlled Word Association test [Benton, 1967]), and visuospatial ability (Rosen Drawing Test [Rosen, 1981]). Age and gender were included as covariates and were removed from the final analysis if they did not significantly contribute to the model.

Mice

Transgenic Mice

Transgenic R(AB) mice were described previously (Abel et al., 1997). The founders were backcrossed to C57BL/6J mice. Mice were maintained and bred under standard conditions, consistent with NIH guidelines and approved by the IACUC. For genotyping, tail DNA was prepared and analyzed by Southern blotting using transgene-specific probes. Littermates were analyzed in all assays.

Imaging Acquisition and Processing

Ten mutants and 10 wild-type littermates underwent MRI analysis. Mice were 6–7 months of age, the average weight (30 g) did not differ between wild-type and transgenic littermates. R(AB) mutants and wild-type mice were imaged in a random order, and genotype was not known to investigators. Three-dimensional gradient-echo sequences were employed. The acquisition matrix was $256 \times 256 \times 16$. Pixel dimension was 78 μm $\times$ 78 μm and slice thickness was 375 μm. TR/TE = 500 ms/21 ms; flip angle = 20; NEX = 1.

Mice were anesthetized with intraperitoneal injections of ketamine. Heart rate and pulse rate were monitored using pulse oximetry (Decker et al., 1989) (Nellcor Puritan Bennet, model NPB-40, reflectance sensor RS-10). Ketamine has relatively little effect on respiratory function, does not suppress cerebral activity, and does not decouple cerebral blood flow and cerebral metabolism. All mice were wrapped in blankets to prevent fluctuations in body temperature.

Histological Methods

Mice were anesthetized with avertin and perfused with 4% paraformaldehyde, postfixed in paraformaldehyde overnight, and sectioned with a vibratome (30 μm sections). Floating sections were transferred to 24-well culture dishes. After hydrogen peroxide (0.3%) incubation for 15 min, sections were washed and then blocked in 10% goat serum, 0.1% Triton X-100 in STE, and incubated with Calbindin-D (Sigma C8666), MAP2 (Sigma M1406), synaptophysin (Sigma S5768), and GAP-43 (Sigma G9264) mouse monoclonal antibodies (1:100 dilution) overnight at 4°C. After washing, the sections were incubated in horseradish peroxidase-conjugated antibody to mouse immunoglobulin (Boehringer) diluted 1:200 in 0.5$\times$ blocking solution for 2 hr. After three washes, DAB was developed development according to the Histomouse-SP kit (Zymed). The Timm staining was performed as described in Sloviter (Sloviter, 1982). The fluoro-Nissl stain was performed according to manufacturer's protocol (Molecular Probes). The acetylcholinesterase staining was performed as described by Koyabashi (Kobayashi et al., 1994). Perls staining for iron localization was performed according to Hill and Switzer, 1984.

Neuronal Counts

Coronal brain sections from four control wild-type mice and four R(AB) transgene littermates were processed for neuron counting. Two sections matched for location between wild-type and transgenic animal were chosen for analysis. Fluoro-Nissl stained hippocampal neurons were photographed with a digital camera on Nikon microscope with a 40$\times$ objective. The resulting photographs were overlayed in Photoshop (Adobe) with a mask with 1000 μm^2 windows. Three randomly chosen windows in the dentate gyrus and the CA1 and CA3 regions of the hippocampus were counted on each section blindly and neuronal counts were matched with the genotypes.

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