Genetics of Childhood Disorders: XLVIII. Learning and Memory, Part 1: Fragile X Syndrome Update

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This series of columns has reviewed how experience and repetition are critical to learning. Exactly how learning occurs at a molecular level within the central nervous system has interested neuroscientists for decades, and several important concepts have emerged. First, the basic pattern of neuronal organization appears to be largely intrinsic to the developing brain. At birth, the human brain already contains the majority of neurons it will have in adulthood. In fact, there are many more neurons present at birth than are actually needed, and approximately half will die through the process of programmed cell death due to lack of use. The axiom "use them or lose them" applies.

A second, related concept is that neuronal activity strengthens immature synaptic connections between neurons, whereas inactive synapses weaken and die away. This process, termed

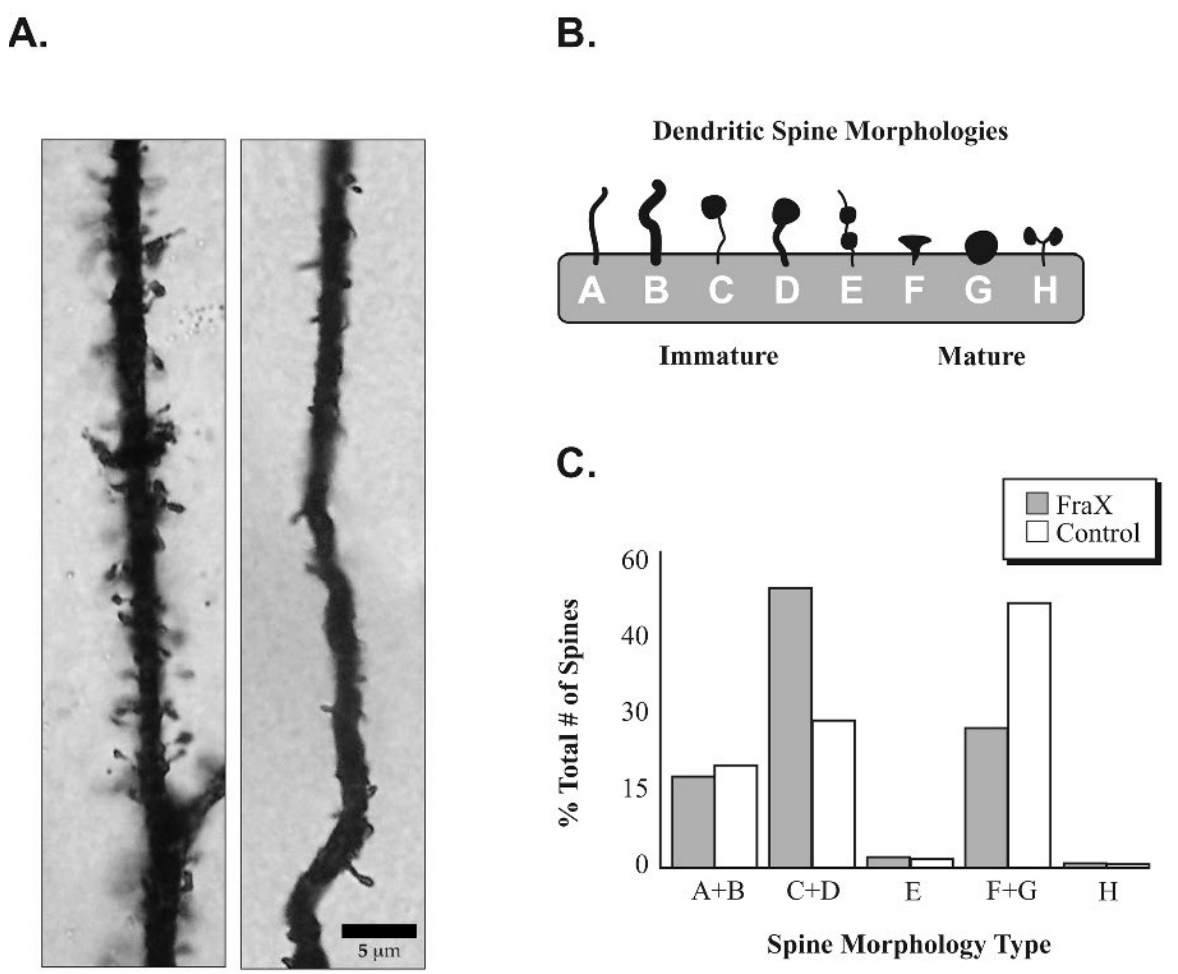

Fig. 1 A: Example of typical spine morphologies on Golgi-impregnated dendrites from a fragile X subject (left) and an unaffected control subject (right). B: Morphology of spines changes with maturation from long and spindly to shorter and broader spines. C: Percentages of different spine shapes seen in fragile X patients compared to normal control brains. Adapted from Irwin SA et al. (2001), Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: a quantitative examination. *Am J Med Genet* 98:161–167. Copyright © 2001 Wiley-Liss, Inc. Reprinted by permission of Wiley-Liss, Inc., a subsidiary of John Wiley and Sons, Inc.

activity-dependent synaptic plasticity, is thought to underlie learning. It requires structural modifications to existing synaptic connections. During development, activity-dependent synaptic plasticity is based on competition for limited amounts of growth factors released by target neurons. More active synapses mature and strengthen. Those that are less active receive insufficient levels of trophic factors and undergo apoptosis.

The external environment exerts its effects on the brain through neuronal activity. Persistent or repetitive neuronal activity leads to structural modifications at exactly those synapses that are being used. Moreover, in regions of the brain that are devoted to learning and memory, repetitive firing at a synapse leads to a striking change in a neuron's ability to respond to action potentials. The synapse becomes more responsive to future action potentials, as the amount of neurotransmitter required to generate a postsynaptic action potential is reduced. This response, called long-term potentiation, is thought to underlie learning and memory in brain regions including the hippocampus, striatum, amygdala, and nucleus accumbens.

Thus two events occur over time with repetitive neuronal activity. The number of synaptic connections between a neuron and its targets increases, and the sensitivity of the individual synapse to neurotransmitters becomes stronger. Both phenomena are thought to underlie the process by which we learn and become fluent in various skills, and both phenomena require new protein synthesis.

This column will review aspects of learning and memory over the next several months. It will cover different forms of memory including declarative, nondeclarative, and working memory. Experts in the field will summarize evidence indicating that different brain regions are responsible for different types of memory. Future columns will outline the molecular events that take place within neurons that lead to the creation, storage, and retrieval of memory. In addition, we will discuss what happens to cognitive processes when mutations occur in genes encoding proteins important for memory formation.

Hundreds of proteins take part in these events, making it likely that many developmental disorders and mental retardation syndromes result from mutations that disrupt one or more of these proteins. It is also possible, although this is more speculative, that certain psychiatric disorders are due to disrupted signaling events involved in the formation and acquisition of memory and its storage and retrieval. Specific phobias and anxiety disorders, and the spectrum of stress disorders, are likely to involve abnormal processing of memories. In the present column, we focus on one aspect of this field, fragile X syndrome, and how discoveries of the genetic basis for this disease have once again linked basic neuroscience with developmental neurobiology.

As mentioned above, activity-dependent synaptic plasticity involves modification to existing synapses. Structural changes occur at both the pre- and postsynaptic sites. For example, where before there was one synapse, two or more synapses form as the neuronal connection responds to synaptic activity. This and additional changes at the postsynaptic site mediate a stronger response to the incoming signal.

How exactly do these structural changes occur? An individual neuron can make contact with 1,000 other neurons, while many additional neurons may synapse on that initial neuron. How does a postsynaptic neuron distinguish among the thousands of potential sites on its dendritic arbor those that require structural modification? This puzzle was partially solved when it was shown that synaptic modification requires new protein synthesis.

Several events are required for new protein synthesis. A signal needs to arrive at the neuron's nucleus. This signal must be transmitted to the nucleus after proteins called transcription factors have been activated. Transcription factors must bind to promoters, which are the regulatory regions on individual genes. Depending on which transcription factors are activated, the promoter region either represses or enhances transcription of that gene. When transcription is enhanced, messenger RNAs (mRNAs) are rapidly transported to the cytoplasm for translation.

Earlier dogma suggested that translation occurred at ribosomes in the cytoplasm around the nucleus, and therefore a considerable distance from the dendritic spine where the initial synaptic input arrived, and where synaptic modification was needed. But how would the newly synthesized synapse-related proteins "know" where to go once synthesized? How are they transported to the correct synapse where modifications are needed?

A new hypothesis was needed, and one soon emerged. Perhaps the idea that new protein synthesis occurred only in the cell body of neurons, close to the nucleus, was incomplete. If the mRNAs themselves were transported to all the spines in the dendritic arbor, then they would be positioned at all postsynaptic terminals, poised, as it were, to be translated into protein after the arrival of the appropriate signal. This hypothesis offered a solution to the problem of getting the newly synthesized proteins back to only the specific spines where they were required.

Over the past several years, considerable data have accumulated in support the idea that mRNAs are themselves transported to spines throughout the dendritic arbor. This allows rapid, local, and selective translation of proteins only at the spines where they are needed, so only a subset of neuron's synapses are modified in response to activation. Once again, however, new questions emerged. How do mRNAs travel to the spines, and how are they regulated? This is where the protein that is mutated in fragile X syndrome comes in.

To understand the molecular biology of fragile X syndrome, it is useful to review the changes in the brains of affected individuals. Overall, autopsy analyses reveal few light microscopic changes. Higher-resolution analysis with electron microscopy shows that the dendritic spines from fragile X patients are abnormal. They are morphologically similar to the spines of imma-

ture, developing brains. The dendritic spines in fragile X individuals are long and thin compared with the short and broad spines of mature cortical neurons (Fig. 1).

We know that neurogenesis and neuronal migration proceed normally in fragile X individuals, suggesting that the pathology occurs later, during synaptic maturation. Apparently, the protein affected by fragile X syndrome or proteins that depend on this protein are necessary for proper synaptic growth and maturation. We now turn to how this story unfolded over the past decade through advances in molecular biology.

Fragile X syndrome is the most common form of inherited mental retardation. It occurs with a frequency of approximately 1:4,000 in males and 1:8,000 in females. The fragile X mental retardation-1 (*FMR1*) gene was cloned more than a decade ago—a major accomplishment. Characterization of the mutation revealed a novel type of genetic mutation called a triplet repeat expansion. This type of mutation was originally identified in Huntington chorea. When the *FMR1* gene was sequenced, and proved to be second example of a triplet repeat expansion, genetic researchers realized that they had discovered a new type of mutation. Since then, triplet repeat expansions have been described in approximately a dozen neuropsychiatric disorders.

These mutations have in common the expansion of an unstable series of nucleotides. In fragile X syndrome, three nucleotides (CGG, cytosine-guanine-guanine) are abnormally repeated over and over again, and they may reach several thousands of nucleotides in length in the most severely affected individuals. The presence of a small number (between 5 and 50) of these triplet repeats is normally present in the *FMR1* gene. In some individuals, however, the trinucleotide repeat expands to 200 repeated CGGs. When this happens, the affected individual is a carrier of a premutation. Then, in the next generation, a dramatic expansion of the repeated sequence among the offspring results in the full-blown clinical syndrome. It is not clear how this expansion occurs or how the premutation in the parent arises.

The consequences of this expansion, however, are dramatic. A chemical modification called methylation occurs throughout the expanded region. In addition, a complex folding to the secondary structure of the DNA molecule occurs. These changes interfere with normal gene transcription. The enzyme that needs to gain access to the DNA molecule to initiate transcription is unable to do so. The net effect is that no message is made and no functional fragile X mental retardation protein (FMRP) is translated.

The next question to interest researchers was how the absence of FMRP leads to mental retardation. Once the gene for fragile X syndrome had been cloned, researchers were able to sequence it and translate the nucleotide sequence into its predicted amino acid sequence. They were then able to determine whether there were any regions within FMRP that were homologous to known proteins. The presence of preserved motifs with known function could suggest a similar function for FMRP that could be tested in the laboratory. Indeed, several amino acid domains were found that were highly homologous to known motifs within other proteins. Three of these domains had previously been shown to bind to RNA molecules, and the proteins that contain them are therefore called RNA-binding proteins.

RNA has several functions within cells. The messages transcribed from DNA consist of mRNA molecules. In addition, the ribosomal factories in the cytoplasm that translate mRNA into protein contain RNA. These RNA populations do not exist by themselves. In the case of mRNAs, a number of proteins bind to them to chaperone them from one compartment to another, and to facilitate their translation into protein. In the case of the ribosomes themselves, RNA-binding proteins provide an organized structure for the protein translation apparatus. In fact, ribosomes contain up to 80 different proteins that come together in a complex that give the ribosomes their form and function. Once again, these proteins have RNA-binding motifs that allow them to associate with RNA molecules destined to be incorporated into ribosomes, and form the scaffold that will accept mRNA molecules and translate them into proteins.

A new series of experiments were designed to test whether FMRP could bind to RNA and, if so, which type of RNA. Researchers showed that FMRP is able to bind to synthetic polymers of RNA in vitro. Immunocytochemical analyses showed that FMRP is predominantly cytoplasmic. Moreover, the protein has two additional amino acid sequences that provide additional clues as to its function. One is a nuclear localization signal present on proteins that are at some point transported into the nucleus. A second amino acid sequence, called a nuclear export signal, has the opposite effect. The presence of both of these signals raised the intriguing possibility that FMRP shuttles RNA messages from the nucleus to the cytoplasm, and then returns to the nucleus to pick up a new RNA message.

At approximately the same time that this work was being done, a patient with a very severe form of fragile X syndrome was discovered. Careful analysis of his *FMR1* gene revealed no triplet repeat expansion. Instead, researchers found a point mutation that changed a single amino acid within one of the putative RNA-binding domains. This mutation resulted in a protein that was unable to bind with RNA molecules. This was strong evidence that the ability of FMRP to bind to RNA was critical to its proper function. Together, these observations suggested that FMRP normally binds to a subset of mRNAs. The absence of FMRP disrupts normal translation of these target messages. Subsequent experiments attempted to test this hypothesis by identifying the subset of messages that bind with FMRP.

With a combination of biochemistry and a newer technology—microarray analysis—two groups have now identified a series of mRNAs that associate with FMRP. Their experimental approach was elegant and worth reviewing. An antibody that recognizes FMRP had already been generated. It could be

used to pull-down, or immunoprecipitate, FMRP from a mixture of brain proteins where FMRP normally resides. Under the right buffer conditions, any proteins or mRNAs bound to FMRP would co-immunoprecipitate. Such experiments revealed a number of messages that co-immunoprecipitated with FMRP. Exactly how these messages were subsequently identified requires a brief description of microarrays.

Microarrays are small chips to which thousands of known DNA sequences have been robotically attached. The DNA sequences are complementary to the mRNAs (thus called cDNAs). Chips are now available onto which 10,000 or more cDNAs have been placed. Researchers can obtain chips for any tissue, including brain-enriched microarrays. The exact location of each cDNA on the chip is known and can be distinguished from any of the other attached sequences.

Researchers investigating the molecular basis of fragile X labeled immunoprecipitated material containing the unknown mRNAs that were associated with the FMRP complex. The mRNAs were labeled with fluorescent tags to make them visible probes, and they were placed (in solution) over the microarray. Because complementary nucleic acid sequences bind very tightly to each other, mRNAs immunoprecipitated from the brain would bind to their complementary cDNAs on the microarray. The cDNAs could now be fluorescently identified by their position on the array. At this point, it becomes straightforward to determine which of the thousands of brain-enriched messages were capable of binding to FMRP. In this manner, a handful of mRNAs were identified. A particularly interesting finding was that the bound messages all had a specific sequence (called a G quartet) that was absolutely required for the message to bind to FMRP.

One of the messages that was identified in this way encodes for a protein called microtubule-associated protein MAP1B. One of the known functions of this protein is to provide structural organization for the synapse. For this reason, current thinking has it that the absence of FMRP leads to a dysregulation of MAP1B expression at the synapse. The absence of MAP1B at the synapse in turn is believed now to contribute to the structural abnormalities seen with electron microscopy in individuals with fragile X syndrome. Their inability to reorganize their synaptic architecture is now believed to be the underlying basis for the cognitive abnormalities characterizing this disorder.

WEB SITES OF INTEREST

http://info.med.yale.edu/chldstdy/plomdevelop/genetics/00febgen.htm
http://info.med.yale.edu/chldstdy/plomdevelop/genetics/99decgen.htm
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