HYPOCRETINS

Integrators of Physiological Functions

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Edited by

Luis de Lecea
J. Gregor Sutcliffe

*The Scripps Research Institute
La Jolla, California*

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Luis de Lecea
The Scripps Research Institute
La Jolla, CA 92037
USA

J. Gregor Sutcliffe
The Scripps Research Institute
La Jolla, CA 92037
USA

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FOREWORD

The first report that rapid eye movements occur in sleep in humans was published in 1953. The research journey from this point to the realization that sleep consists of two entirely independent states of being (eventually labeled REM sleep and non-REM sleep) was convoluted, but by 1960 the fundamental duality of sleep was well established including the description of REM sleep in cats associated with “wide awake” EEG patterns and EMG suppression. The first report linking REM sleep to a pathology occurred in 1961 and a clear association of sleep onset REM periods, cataplexy, hypnagogic hallucinations and sleep paralysis was fully established by 1966.

When a naïve individual happens to observe a full-blown cataplexy attack, it is both dramatic and unnerving. Usually the observer assumes that the loss of muscle tone represents syncope or seizure. In order to educate health professionals and the general public, Christian Guilleminault and I made movies of full-blown cataplectic episodes (not an easy task). We showed these movies of cataplexy attacks to a number of professional audiences, and were eventually rewarded with the report of a similar abrupt loss of muscle tone in a dog. We were able to bring the dog to Stanford University and with this as the trigger, we were able to develop the Stanford Canine Narcolepsy Colony. Breeding studies revealed the genetic determinants of canine narcolepsy, an autosomal recessive gene we termed *canarc1*. Emmanuel Mignot took over the colony in 1986 and began sequencing DNA, finally isolating *canarc1* in 1999.

As the leading instigator of the early efforts, I am content that the considerable outlay of funds to house and feed a large colony of narcoleptic canines for twenty years has paid off, and paid off handsomely I might add. This book is mainly about what has happened and is happening after the isolation of *CANARC1*. For individuals interested in sleep disorders, circadian rhythms, sleep regulatory processes, and other brain mechanisms, this book is a must read.

William C. Dement, M.D.
Stanford University,
Sleep Disorders and Research Center,
Palo Alto, CA

PREFACE

1. HOW IT STARTED

Although our publication of the discovery of the two peptides we named the hypocretins did not occur until 1998, the road to their discovery began in the spring of 1979. One of us (JGS) was completing postdoctoral studies with Richard Lerner at the Scripps Clinic and Research Foundation (now The Scripps Research Institute) following doctoral work performed under Wally Gilbert at Harvard, where the main thesis efforts had been in scaling up the DNA sequencing procedures developed by Gilbert and Allan Maxam. Those studies, which were described in a *Reflections* piece in *TIBS*,¹ represented the first time DNA analysis was used to determine the sequence of a protein in the absence of peptide sequence information from the protein itself. Thus, one learned first hand about the superior accuracy and rapidity of carefully collected DNA-based sequence information and the possibility that one could use the methods to determine the sequences of previously unrecognized genes for which neither proteins nor RNAs were known. This was a quarter of a century before mammalian genome sequences began to appear.

In Lerner's laboratory, JGS was working with Tom Shinnick on determining the first retrovirus genome sequence, that for Moloney murine leukemia virus.² Lerner knew of his long-term interest in neurobiology. The eminent neurobiologist Floyd Bloom, then a professor at the neighboring Salk Institute, asked Lerner if the technologies JGS had brought to Scripps could be used to study the brain. Bloom had just read the report describing the cDNA cloning of proopiomelanocortin (POMC),³ and was particularly interested in finding out about undiscovered peptide neurotransmitters. Lerner passed along the question.

JGS answered that, since so little was known about the molecular operation of the brain, a rational approach would be to construct cDNA libraries from brain mRNA. Individual cDNA clones could then be isolated from such libraries and their nucleotide sequences determined, thus allowing the amino acid sequence of the protein encoded by the corresponding brain mRNA to be conceptually translated. cDNA cloning had recently been developed, and it was the route by which clones were obtained for mRNAs encoding particular, already known proteins, such as POMC. cDNA libraries represent all of the mRNAs expressed in the tissue from which the sample was isolated and, thus, such libraries could inform us about the complete protein set, including those proteins that were not yet identified. But that led to an obstacle: how could we use the conceptual protein sequences translated from the cDNA sequences to learn about the putative

proteins themselves? The proteins would not have been seen previously, and there were few protein sequences to which to compare the new sequences. The solution was to prepare synthetic peptide fragments of the proteins and use these to elicit antibodies that would react with both the peptides and the novel protein itself, thus facilitating its biochemical and anatomical characterization. The first opportunity to apply this approach on a putative protein in the Moloney virus sequence.⁴

Bloom, his then-postdoctoral fellow Rob Milner and JGS began a collaboration to test these ideas. The first effort was to use northern blot hybridization to characterize the mRNAs corresponding to clones in a brain cDNA library. By analyzing the size, abundance and tissue distributions of the mRNAs corresponding to nearly 200 clones isolated randomly from a rat brain cDNA library,⁵ the team calculated that the 10^8 to 2×10^8 nucleotides of mRNA complexity expressed by brain corresponded to 20,000 to 40,000 distinct mRNAs, numbers that compare favorably with modern estimates since entire genome sequences have been solved. Of these, approximately 65% were enriched in the brain compared to peripheral tissues. Most were of low abundance, on the order of one part in 10^5 . The team raised antisera directed against synthetic peptides corresponding to one of the first partial putative brain protein sequences determined, and used these to detect the protein in brain extracts and to conduct a preliminary anatomical description of the protein later shown to be myelin-associated glycoprotein.⁶

These early studies represent the beginning of what have since come to be known as open-system approaches to mRNA expression analysis: mRNAs are detected because of their property of being expressed in the tissue sample isolated for study. Refinement of this approach led to the discovery of the hypocretins. The notion that the tools of molecular biology could be used to address fundamental questions about the operation of the mammalian brain was controversial at the time, but is one that no one today would argue. From the sequences of brain cDNAs, we learn about the nature of brain proteins. The cDNA clones also allow the study of the brain genes themselves and, with the advent of transgenic and knockout technologies, allow the power of genetic analysis to be brought to bear on the central nervous system, permitting a forceful molecular dissection of CNS physiology.

2. REFINEMENTS

The team was inspired by the success of its initial collaborative studies, which were among the first applications of what would later become known as genomics to neurobiology. Bloom, Milner and John Morrison moved from Salk to Scripps, and together with JGS initiated a program project aimed at expanding the effort. The Sutcliffe laboratory's role in the program was that of discovery of novel brain-specific proteins.

Which of the thousands of brain-specific mRNAs to characterize? The early studies demonstrated that many neuronal mRNAs exhibited differential distributions within the CNS; however, their expression was generally not restricted to a few discrete loci that could be attributed to specialized functions, but rather was variegated across the CNS. The studies therefore evolved to focus upon the identification of mRNAs that show a high degree of regional enrichment within the CNS. The logic that motivated this focus was that mRNA molecules with restricted expression were likely to encode proteins that are singularly associated with the unique functions of the cells that contain them and, perhaps, might be preferentially associated with particular physiological or behavioral processes

compared with molecules with more general patterns of expression. Hence, their functional roles might be more transparent to investigation. Furthermore, such molecules might, in the future, provide highly specific targets for pharmaceuticals that would act only at the restricted site of target expression.

In order to enrich our libraries for such mRNAs, and the team turned to subtractive hybridization. Subtractive hybridization refers to a series of methodologies that compare cDNA sequences from one RNA sample, the target, with those from a second sample, the driver. Nowadays, the two complementary, antiparallel strands of cDNA can be produced in either of their single-stranded orientations, sense or antisense, using modern cloning and enzymological procedures. When sense strand from the driver is supplied in great excess over antisense strand from the target and these reagents are coincubated under conditions that favor the formation of double-strand hybrids, most of the mass of those sequences that the target and driver have in common becomes double stranded, whereas sequences from the target population that are absent from the driver population remain single stranded. The single-stranded material is isolated and used to create cDNA libraries enriched for target-specific mRNAs or enriched, radioactive probes for screening libraries. This methodology, originally developed by Timberlake⁷ for studies on gene expression in fungi, has been progressively improved in the ensuing decades to a degree that it has allowed identification of mRNAs selectively expressed within complex mammalian nervous tissue.

Gabe Travis joined the group and introduced mixed-phase methods for increasing the apparent concentrations of the target and driver nucleic acids, thus vastly increasing the extent of their hybridization and hence enhancing enrichment for target-specific sequences.⁸ He and Miles Brennan applied the improved subtraction method to isolate mouse retinal photoreceptor-specific mRNAs, including that corresponding to the product of the *retinal degeneration slow* gene, whose human homologue accounts for a considerable portion of heritable late-onset blindness.⁹ Joe Watson had success in the identification of forebrain-enriched mRNAs, including those RC3/neurogranin¹⁰ and G protein γ .¹¹ Nevertheless, the method, although occasionally effective, was cumbersome and inconsistent.

These shortcomings were overcome with the advent of PCR. Hiroshi Usui, with design input from Mark Erlander, developed a method (simplified here) in which the target cDNA is cloned into a vector that introduces PCR primer binding sites on both sides of the cDNA insert. After hybridization with the driver, the single-stranded target is PCR amplified and cloned. The refined method, called directional tag PCR subtractive hybridization,¹² was used to prepare cDNA libraries enriched for clones of mRNAs specific to the striatum. Screening the large number of clones produced required high-throughput in situ hybridization. LdL developed a free-floating section method and brought anatomical expertise to the group.

As a result of implementing the refined subtraction method and high throughput in situ hybridization, we identified cortistatin, a neuropeptide of the somatostatin family expressed in the neocortex and hippocampus.¹³

3. ON TO THE HYPOTHALAMUS

With this powerful arsenal of new techniques and success record, we were prepared to search for new peptide neurotransmitters, and turned to the hypothalamus as a likely place to find them. We were joined in the search by Kaare and Vigdis Gautvik, who

performed the subtractive hybridization studies that led to finding a clones for what later became known as the hypocretins.^{14,15} Those studies are described in Chapter 1. Patria Danielson and Pam Foye conducted the bulk of the blotting and sequencing experiments; Tom Kilduff and Cristelle Peyron most of the neuroanatomical characterizations.

These studies were not big biology, but were so cross disciplinary as to demand extensive collaborations. The trail that the team embarked upon in 1979 has been consistently productive, but finding the hypocretins has been particularly gratifying, in part because finding such proteins was a large part of its original impetus.

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J. Gregor Sutcliffe and Luis de Lecea
 Department of Molecular Biology
 The Scripps Research Institute
 La Jolla, CA 92037

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