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Combining genetic and genomic approaches to study mood disorders

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Abstract

Recent technological advances in genetic manipulations and DNA microarrays are profoundly altering the landscape of biological research, offering opportunities to investigate biological questions that were only dreamed of a few years ago. With this revolution comes the hope of being able to tackle some of the more arduous challenges that the central nervous system has presented to the research community. Specifically, a major goal in the study of neuropsychiatric disorders has been to identify underlying mechanisms of brain dysfunction with the expectation that these insights may allow a better diagnosis, prevention and effective treatments for these disorders. For the most part, treatments of these disorders have relied on serendipitous discovery of pharmacological entities with therapeutic efficacy, while the causes of the disorders have remained unknown. The serotonin system, and the serotonin$_{1A}$ (5-HT$_{1A}$) receptor in particular, have been under intense investigation, mostly due to the fact that serotonergic drugs that directly or indirectly affect the 5-HT$_{1A}$ receptor, are effective therapeutic agents in treating patients with various neuropsychiatric disorders, including anxiety and depression. Genetic deletion of the receptor in mouse results in increased anxiety, thus supporting an active role for this receptor in mood regulation. However, the analysis of genetic deletion experiments can be confounded by hidden developmental roles of the missing receptor, by adaptive compensatory mechanisms, as well by the fact that the genes or gene products that are responsible for the cellular and molecular aspects of the phenotype may be several steps removed from the genetic intervention. Here, we present a combined methodological approach of tissue specific and conditional genetic manipulations, with large-scale search for altered gene expression, as an experimental framework to investigate the role of genes with complex functions and/or complex expression patterns. The 5-HT$_{1A}$ receptor is used as a model of gene product with complex functions and distributions, and as a prototypical system to which these new genetic approaches are currently being applied.

Keywords: Serotonin receptor; Genetic; Genomic; Depression; Anxiety; Knockout; Microarray

1. Introduction

In order to elucidate mechanisms of brain disorders, as well as to search for new targets for pharmacological agents with therapeutic activity, studies have concentrated in two general directions: first, a search for markers of abnormal functions in patients, including pathophysiology, brain activity, altered protein or RNA functions, DNA mutations, genomic loci, etc., and second, a pharmacological approach that has benefited from the availability of compounds that target specific sites within the central nervous system and affect either the development or treatment of the disorder. With regard to anxiety and depression, the analysis of results obtained from both approaches has yielded conflicting results. For instance, the serotonin system is known to play a key role in these disorders, but its mechanism of involvement remains unknown. Pharmacological agents that target components of the serotonin system have been successful at treating some of the symptoms of these disorders, possibly by regulating the processes that appear to have been dysregulated during the course of the disease. Tricyclic antidepressants (TCAs) and subsequently, serotonin selective reuptake inhibitors (SSRIs), such as fluoxetine or Prozac, efficiently target the serotonin system by increasing the availability of this monoamine. However, the multiplicity of receptor subtypes and of interaction with other neurotransmitters systems, as well as the impact of the environment and genetic factors, have limited a more refined cellular or molecular understanding of the actual mechanisms that are involved in the disease process.

Genetic manipulations in mice offer an absolute specificity of intervention that is not available with drugs. Until recently, these direct genetic approaches had mostly consisted of ablating single genes from the mouse genome (knockout mice, KO), using homologous recombination in

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embryonic stem cells, and by studying the consequences of the lack of a single gene product on the physiology and behavior of developing and/or adult mice (Bronson and Smithies, 1994; Koller and Smithies, 1992). Accordingly, the study of mice with designed mutations in their genomes has helped researchers in deducing important contributions of serotonin receptors to aspects of psychiatric disorders and drug abuse (reviewed in Murphy et al., 1999). For instance, mice lacking the 5-HT_{1B} receptor were reported to be more aggressive (Saudou et al., 1994) and to be more sensitive to the effects of cocaine administration (Rocha et al., 1998). With regard to anxiety, several valuable new animal models have been created by targeted disruption of genes within candidate neurotransmitter systems. Genetic deletion of the 5-HT_{1A} receptor in mouse results in an increased anxiety phenotype (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998). Disruptions at several levels of the corticotropin-releasing factor (Koob and Heinrichs, 1999) system either increase or decrease anxiety levels. Genetic manipulations of the GABAergic system have also confirmed the potent role of this neurotransmitter in the modulation of anxiety-related behaviors in mice (Crestani et al., 1999; Low et al., 2000).

However, when the function of a gene is modified in an otherwise intact organism, it is important to remain critical as to the possibilities of redundancy, compensations and hidden developmental roles that may confound experimental observations (Gingrich and Hen, 2000). Furthermore, the inherent complexity in the pattern of expression and in the function of some genes and gene products may preclude a refined analysis of the mutant phenotype. For instance, the 5-HT_{1A} receptor is expressed in two different neuronal populations where it exerts different functions (reviewed by Hamon, 1997). In the midbrain, 5-HT_{1A} receptors are expressed in the somatodendritic compartment of serotonergic neurons (presynatptic autoreceptors), where it regulates the output of the serotonin system by feedback inhibition. The 5-HT_{1A} receptor is also expressed in several postsynaptic non-serotonergic neuronal populations (postsynaptic heteroreceptor), where it performs a different role in brain regions that are targeted by the serotonin system. Deleting this receptor may therefore yield important information as to its overall contribution to brain function but will not allow discriminating between its pre- and postsynaptic roles. Therefore, in order to distinguish between separate functions for a same gene product and to address the question of compensatory mechanisms, targeting constructs for homologous recombination have been modified to include elements that permit a temporal and spatial control of the expression of the targeted gene, instead of 'constitutively' blocking its transcriptional activity.

Equally important as the selectivity of the genetic intervention, is the analysis of the phenotype of the mutant mice. For many gene products where specific inhibitors or antagonists are lacking, as well as for orphan receptors, knockout studies have been highly successful at associating new functions with these genes, with the only caveat being the need to infer function from the absence of this function. On the other hand, for gene products with existing inhibitors or antagonists, knockout studies have not always mimicked pharmacological blockades, suggesting the occurrence of compensations by functionally related systems. In view of these observations, a marked appreciation of the contribution of specific gene products in setting proper neural networks during development is rapidly growing for several neuropsychiatric disorders. Consequently, it has become crucial to be able to describe the nature and extent of these adaptations so that the expression of the phenotype of genetically modified mice may be explained not only by the lack of a gene product, but by the precise changes that may have happened at the molecular, cellular and neural network levels in the mutant organism. The identification of these 'effectors' of the mutant phenotype actually accounts to a 'reverse' genetic approach, where the emphasis is on identifying genes whose altered expression correlate with the observed phenotype. This approach can potentially identify new or unsuspected participants in the expression of the phenotype. In the context of animal models of neuropsychiatric disorders, such 'effector' genes could represent potential new targets for pharmacological treatments of the symptoms of these disorders. Traditionally, people have relied on a candidate gene approach to identify such 'effectors', where the genes, enzymes, receptors, etc., that are the most likely to be functionally related to the missing protein, are investigated. However, these lines of study are severely limited by the extent of our current knowledge of the function of most genes and their implications in various biological systems and pathways. Recent developments in DNA microarray technologies permit a systematic approach that has begun to have a profound impact on biological research (Lander, 1999). The possibility of obtaining information on expression levels of tens of thousands of genes in a simultaneous and unbiased way strongly suggests that new genes and pathways will be identified in biological screens for specific activities.

Here, we describe the current technology and experimental approaches that have been undertaken in our laboratory to search for altered gene expression that may be responsible for the observed phenotype. Currently, we are combining tissue specific and conditional genetic manipulations, with large-scale search for altered gene expression, as an experimental framework to investigate the role of genes with complex functions and/or complex expression patterns. The possibility to examine the behavioral consequences of manipulating particular genes in specific brain regions, and to investigate in parallel the expression levels of thousands of genes, may yield invaluable insights into mechanisms of brain function. The 5-HT_{1A} receptor is used as a model of gene with complex functions and expression pattern, to illustrate the advan-

tages and limitations of conventional knockout studies and to outline some of the investigational opportunities that are offered by these new approaches.

2. Genetic manipulations: regional specificity and conditional expression

In classical knockout studies, the function of a gene is disrupted by removing part of the coding region of the gene and replacing it with a neomycin-resistance DNA cassette by homologous recombination in embryonic stem (ES) cells (Fig. 1). The antibiotic-resistance cassette is then used to select recombinant cells. Homologous recombination typically happens on a single chromosome and ES cells that are heterozygous for the mutation are injected into mouse blastocysts. The recombinant ES cells incorporate randomly into blastocysts which are re-implanted into pseudopregnant female mice. Pups derived from these blastocysts will carry cells that originated from both the native and the recombinant ES cells. At this stage of development, all blastocyst cells and injected recombinant cells are embryonic pluripotent stem cells which divide and give rise to all cell types in the adult animal. These 'chimeric' animals are visually recognizable, as their fur coat reflect the mixture of brown/agouti hair that originated from the 129SvJ mouse strain that was used to derive recombinant ES cells, and the black hair from the C57 mouse strain blastocyst cells. If the recombinant ES cells gave rise to cells of the reproductive system, the mutation will be transferred to the next generation. Eventually, mutant mice are bred to homozygozity for behavioral testing and molecular characterization of the effects of the mutation on the organism. Such mutant mice exhibit a total lack of expression of the mutated gene throughout their lifetime in all tissues, and are referred to as 'constitutive' knockout.

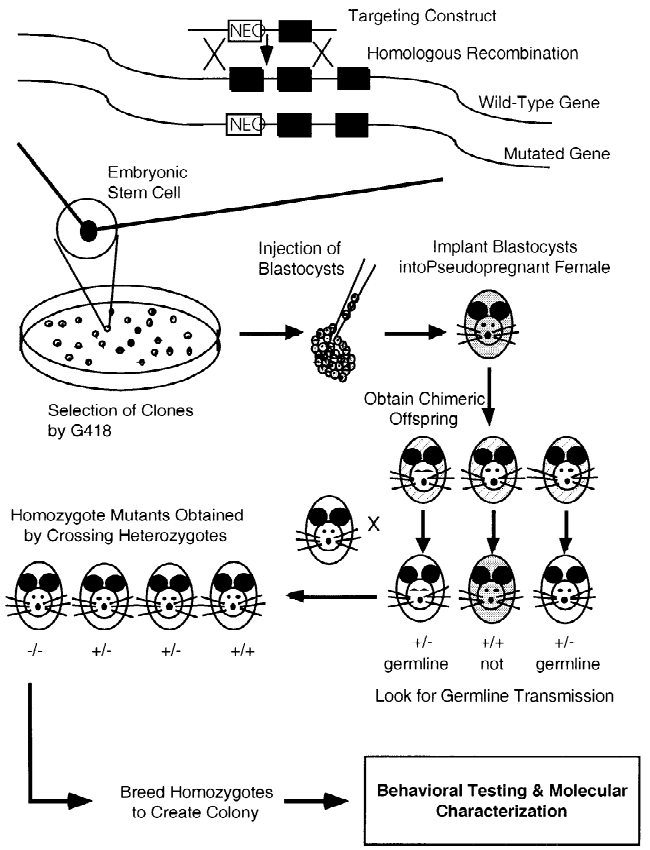

Fig. 1. The making of a knockout mouse. The function of a gene is disrupted by removing part of the coding region of the gene and replacing it with a neomycin-resistance DNA cassette (NEO) by homologous recombination in embryonic stem (ES) cells. Recombinant ES cells are injected into mouse blastocysts which are re-implanted into pseudopregnant female mice. 'Chimeric' pups are screened for germline transmission. Mutant mice are bred to homozygozity for behavioral testing and molecular characterization (see text for detailed comments). +/+, wild-type; +/−, heterozygotes; −/−, homozygotes.

Using this approach of selective genetic deletion, hundreds of knockout mice have been created in the last decade, yielding large amount of biological information on numerous genes and giving rise to countless new models of human disorders. With regards to the 5-HT_{1A} receptor, human post-mortem studies and pharmacological experiments have suggested a link between decreased receptor function and neuropsychiatric disorders, including anxiety and depression (Coplan et al., 1995; Julius, 1998). To test this hypothesis, three laboratories have independently created knockout mice for this receptor (5-HT_{1A} KO) and have all reported a phenotype of increased anxiety-related behaviors in the mutant mice (Parks et al., 1998; Ramboz et al., 1998; Heisler et al., 1998), thus, confirming the critical role of this receptor in the modulation of anxiety. However, since the receptor is absent from both presynaptic and postsynaptic compartments, these studies do not allow the discrimination between the relative contribution of these two separate pools of receptors to the phenotype. From a mechanistic point of view, it will be crucial to achieve this level of discrimination. For instance, antidepressant drugs have been suggested to require a desensitization of pre- and not postsynaptic 5-HT_{1A} receptors to increase serotonin levels (Artigas et al., 1996; Blier and de Montigny, 1994), while tonic activation of postsynaptic receptors may confer therapeutic effects (Haddjeri et al., 1998). The investigation of both hypotheses has important consequences for the understanding of the disease process and for future drug development. In general, a refined analysis of genes with complex patterns and multiple functions will require such a degree of discrimination, in order to better understand their involvement in normal physiology and in the etiology of neuropsychiatric disorders. Taken together, constitutive knockout experiments have brought about considerable progress in the analysis of gene function, however a lack of spatial or temporal gradation in the effects of the genetic manipulation have limited the investigational scope of these studies (Lucas and Hen, 1995; Stark et al., 1998).

In order to address these questions, new targeting construct were developed that allow control over the level of expression of a particular gene in a temporal and/or regional fashion. These new approaches circumvent some of the complications that are inherent to the constitutive deletion of a gene in germ lines. Fig. 2 displays the strategies that have been employed in our laboratory to achieve temporal and regional control over the 5-HT_{1A} receptor. In the present design, a DNA sequence consisting of a STOP cassette is introduced immediately downstream of the transcription initiation site of the gene. This STOP cassette efficiently shuts down all transcriptional activity, resulting in a null mutant for the 5-HT_{1A} receptor. The DNA cassette that was introduced in the locus, or 'knocked-in', bears two additional sets of features that were designed to reawaken the transcriptional activity of the gene.

First, the minimal operator sequence (tetO) of the bacterial tetracycline system serves as a docking site for the tetracycline transactivator (tTA) transcription factor (Bujard, 1999), which now efficiently drives the transcription of the downstream 5-HT_{1A} receptor gene. The tTA protein is produced only in bacteria, but can be introduced in the mouse genome under the control of a chosen promoter. In this instance, the expression of the tTA protein is driven, in a separate line of transgenic mice, to several forebrain regions including the hippocampus, cortex, amygdala and striatum, under the control of the Ca^{2+}-calmodulin dependent kinase II (CamKinase II) promoter (Mayford et al., 1996). When cross-bred with 5-HT_{1A} STOP receptor knock-in mice, double transgenic mice express the 5-HT_{1A} receptor under the control of the tTA protein in the brain regions described above. Thus, the 5-HT_{1A} receptor is 'rescued' specifically in postsynaptic brain regions, as tTA is not expressed in the raphe nuclei in these mice. Furthermore, the binding of tTA to its operator sequence, tetO, can be prevented by tetracycline. When provided in the food or drinking water, doxycycline (DOX), a tetracycline analog, efficiently binds to tTA, preventing binding to tetO and shutting off all transcriptional activity at the 5-HT_{1A} receptor locus. Altogether, the tTA–tetO system represents an inducible system that allows a dual control over the expression of the receptor: first, the regional distribution is determined by the specificity of the tTA promoter, and second, the time and duration of expression is regulated by the presence or

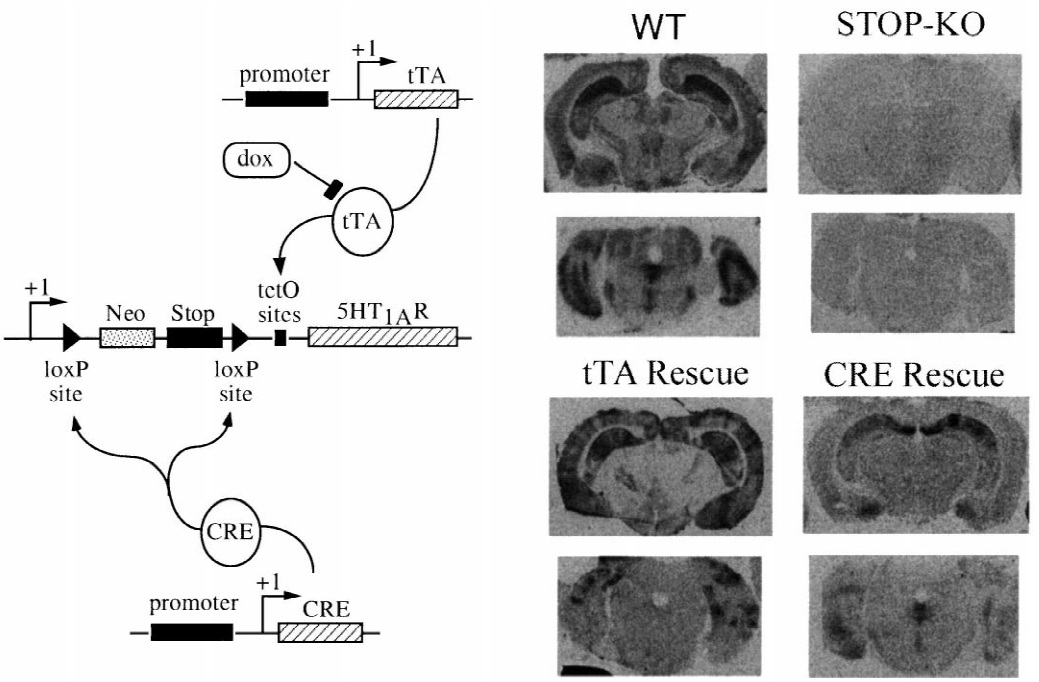

Fig. 2. Temporal and regional genetic manipulations of the 5-HT_{1A} receptor. A DNA sequence consisting of a neomycin resistance cassette and a STOP sequence, both flanked by LoxP sites, as well as a minimal tetracycline operator sequence (tetO), are introduced by homologous recombination in embryonic stem cells (knock-in) immediately downstream from the site of initiation of transcription. The recombinant DNA cassette efficiently shuts down all transcriptional activity, resulting in a null mutant. The transcriptional activity of the gene can be reawaken by the binding of the tetracycline transactivator (tTA) transcription factor. The tTA protein is provided by crossing knock-in mice with transgenic mice that express tTA under the control of a chosen promoter. When doxycycline (DOX) is present, the binding of tTA to its promoter tetO is prevented and the transcriptional activity is shut off. The STOP cassette can also be removed by CRE-mediated LoxP excision, thus re-activating the transcriptional activity of the 5-HT_{1A} receptor under its endogenous promoter. On the right panels, ^{125}I-MPPI autoradiographs depict 5-HT_{1A} receptor binding at two anatomical levels: hippocampus (postsynatic heteroreceptors) and raphe (presynaptic autoreceptors). WT: normal 5-HT_{1A} receptor levels; STOP-KO: the STOP cassette efficiently blocks 5-HT_{1A} receptor transcriptional activation; tTA Rescue: CAMKII-tTA rescues receptor expression in hippocampus, but not in raphe; CRE Rescue: CRE (under the control of a heat-shock protein promoter) deletes the STOP cassette and re-activates receptor expression in both pre- and postsynaptic brain regions.

absence of DOX in the system. For instance, the 5-HT_{1A} receptor can now be induced only in post-synaptic brain regions either during development or in the adult animal, thus allowing to separate developmental contributions of the receptor in specific brain regions from its adult role. Additional transgenic lines with promoters driving the expression of tTA in midbrain pre-synaptic brain regions or with more restricted forebrain patterns of expression are currently being developed.

The second conditional feature that was engineered into the recombinant DNA cassette at the 5-HT_{1A} receptor locus is a set of LoxP sites, flanking the NEO and STOP sequences. LoxP sites are short palindromic DNA sequences that are recognized by the bacterial CRE recombinase protein (Sauer, 1998). When expressed at sufficient level, CRE excises the DNA sequence that lies in between two loxP sites and ligates the two sites into one. Removal of the STOP cassette recreates the normal structure of the 5-HT_{1A} receptor promoter (with the exception of the additional 34 base pairs of the unique LoxP site) which reactivates the transcriptional activity of the 5-HT_{1A} receptor according to its endogenous pattern of expression. Similarly to the tTA protein, CRE is provided by crossing 5-HT_{1A} receptor knock-in mice with a transgenic line that expresses CRE under the control of a chosen promoter. Importantly, the 5-HT_{1A} receptor will only be expressed in the brain regions that lie at the intersection between the pattern of CRE expression and the endogenous pattern of the receptor. In contrast to the tTA–tetO system, where tTA induces transcription regardless of the endogenous pattern of expression of the downstream gene, activation with the CRE system requires LoxP excision of the STOP cassette *and* activation of the endogenous promoter. Therefore, the CRE system may activate a gene only in cells that normally would express this gene. On the other hand, the tTA–tetO system results in a combination of endogenous pattern and ectopic expression, when the tTA promoter is also active in cells that usually do not express the targeted gene. This particular feature of the tTA–tetO system is also used to overexpress gene products with strong promoters either in native or ectopic brain regions. Finally, an additional difference between the two systems resides in the fact that the excision by CRE recombinase is irreversible and thus the CRE system, unlike the tTA–tetO system, cannot be applied to modulate expression over time.

Taken together, these tissue-specific and conditional genetic manipulations provide better tools to refine our understanding of the contributing roles of complex gene products, such as the 5-HT_{1A} receptor, to normal physiology and indirectly to mechanisms of neuropsychiatric disorders.

3. Genomic approach to phenotype analysis

The phenotypic expression of mutant mice often do not fully mimic pharmacological blockade or inhibition of the gene product. For instance, mice lacking the 5-HT_{1B} receptor subtype demonstrated an increased locomotor response to cocaine and a higher tendency to self-administer the drug (Rocha et al., 1998), while injection of a 5-HT_{1B} receptor, GR127935, decreased the locomotor effects of cocaine and had no effects on self-administration (Scearce-Levie et al., 1999). Likewise, in mice lacking the 5-HT_{1A} receptor, the profile of anxiety-related behaviors does not fully recapitulate antagonist blockade of the receptor (Gross et al., 2000). These observations suggest that genetic deletion does not equate with pharmacological blockade of the receptor and that the observed phenotype may represent the expression of compensatory events that are several steps removed from the original genetic manipulation. Accordingly, the absence of the receptor during development could lead to either a lack of proper maturation of specific neural networks (deficit phenotype) or to adaptations in other systems (compensated phenotype) that can confound the interpretation of the subsequent phenotype. Therefore, these studies highlight the necessity to investigate the occurrence and nature of possible changes in other genes and systems, in order to better understand the exact mechanisms that are responsible for the observed phenotype.

Recent developments in microarray technologies have helped focus the attention on RNA expression levels as markers of cellular physiology (Fig. 3). Relative changes in RNA abundance are monitored between samples and assumed a priori to represent changes in function or activity of the cell. Current methodology for investigating gene expression includes protocols that are based on unbiased RNA transcript sampling or on knowledge of defined sets of genes and expressed sequenced tags (ESTs). RNA transcript sampling includes differential display and serial analysis of gene expression (SAGE). These approaches are sensitive and could, in theory, detect any expressed transcript. However, they require time-consuming cloning, sequencing and analysis. On the other hand, the development of DNA microarrays has significantly enhanced the capacity of screening large sets of genes and ESTs. The array technology is essentially an extension of the Southern hybridization. The novel modification is that, unlike standard Southern hybridization, in which the labeled probe is free in solution and the target DNA is fixed on a solid phase, microarray methodology reverses the position of the target DNA and the probe (Southern et al., 1999). Thus, it is more like a dot blot where the expression levels of multiple genes displayed a probes on a fixed matrix are investigated simultaneously in a single hybridization reaction (Duggan et al., 1999). Adaptation of photolithographic masking techniques used in semiconductor industry and of light-directed chemical synthesis processes, has greatly extended the power of this approach. With the aid of computer-based analysis of gene sequences, it is now possible to synthesize and analyze up to 400 000 distinct oligonucleotides on a single array that is not larger than one and a half square centimeters (Lockhart

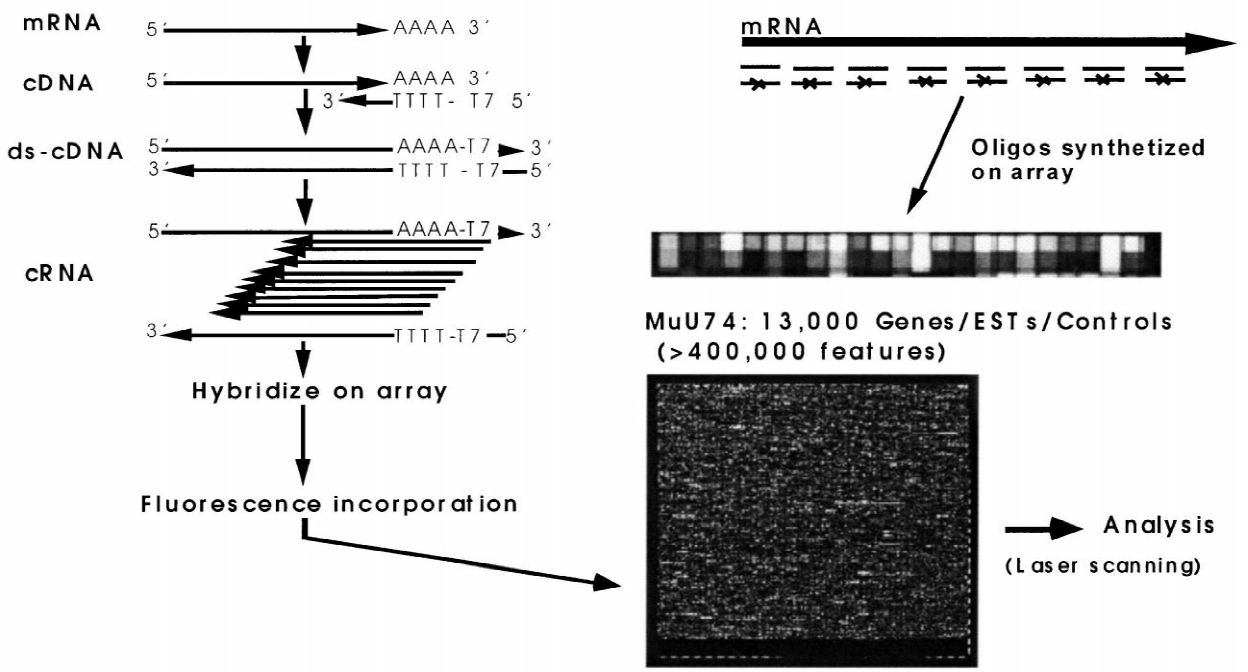

Fig. 3. Oligonucleotide DNA microarray. On Affymetrix oligonucleotide DNA microarray, each gene is represented by 16–20 specific complementary 25-mer oligonucleotides. An equal number of mismatch oligonucleotides controls for the specificity of the signal. All oligonucleotides are synthesized directly onto the glass slide. For the latest generation of murine microarray, 13 000 genes, ESTs and controls are represented by more than 400 000 features on a single microarray (U74 series). To prepare samples, total or polyA RNA is extracted and converted into ds-cDNA. A copy RNA or cRNA is then transcribed in vitro and biotinylated. This cRNA is then hybridized onto the microarrays. Fluorescence is coupled to hybridized probes by avidin and biotin antibody-coupled fluorophore. Finally, a laser scan is obtained for the analysis. Two kinds of analysis are performed. On a single chip analysis, a gene is determined to be present or absent, depending on its pattern of hybridization across all features representing this transcript on the array. An estimate of the expression level for each transcript is provided by the average of the difference in signal intensity between hybridization at perfect match and mismatch oligonucleotides across all 16–20 pairs. In a pairwise comparison, the efficiency of hybridization at each oligonucleotide is compared between two samples that were hybridized on two separate arrays. For both types of analyses, quantitative (average differences) and qualitative (present, absent, increased or decreased) calls are the results of decision matrices that take into account several parameters and metrics that describe the hybridization of each transcript.

et al., 1996). To increase the specificity of hybridization reactions that interrogate the whole transcriptome (set of all RNAs expressed in the biological sample) in a single reaction, the knowledge of sequence information stemming from the effort to sequence whole genomes was used to design specific oligonucleotide for thousands of genes. As little as 200–300 bases of gene or EST sequences are sufficient to create 15–20 non-overlapping, unique 25-mer oligonucleotides, which represent specific probes for this given sequence. On oligonucleotide microarrays, each gene is represented by a set of 20 perfectly matched (PM) and by 20 adjacent mismatched (MM) 25-mer oligonucleotides. The use of probe redundancy to assess the expression level of a specific transcript greatly improves the signal to noise ratio (efficiency of hybridization are averaged over 20 probes), increases the accuracy of RNA quantification (removal of outlier data) and significantly reduces the rate of false positives. Such arrays are made commercially available through Affymetrix, Inc. (Santa Clara, CA). Although not included in this review, cDNA clones or amplified PCR fragments can be directly spotted onto glass or membrane solid support. cDNA microarrays are either custom-made from library of clones or commercially available (Duggan et al., 1999).

For sample preparation, RNA is extracted, converted into double-stranded cDNA. A biotinylated complementary RNA (cRNA) is then transcribed in vivo, using a RNA polymerase T7 promoter site which was introduced during the reverse-transcription of RNA into cDNA. After fragmentation in pieces of 50–200 bases long, the labeled cRNA sample is hybridized onto the microarray, using standard protocols with the Affymetrix microarray oven and fluidics station. A high-resolution image of the hybridization pattern on the probe array is obtained by laser scanning, and fluorescence intensity data is automatically stored in a raw file. A large number of built-in internal controls allow to assess the quality of the overall process. Quality control criteria include: visual inspection of the scan, quality of the reverse transcription reaction, detection level of spiked bacterial control genes, quality of the fluorophore incorporation, noise and background levels. Based on the intensity of the fluorescent signal across the PM cells and the ratios of PM to MM cells, a detection call (absent, marginal or present) and a relative intensity level are attributed to each gene. The data that is obtained for each gene or EST on the array is then normalized and scaled to an averaged fixed pre-determined value. Likewise, the intensity of hybridization at each individual

oligonucleotide can be compared between two samples hybridized on separate arrays. These pairwise comparisons are performed to determine the relative change in abundance for each transcript between two experimental conditions. These values (absolute levels and relative changes) are then used for large-scale comparison analysis.

In addition to straightforward search for replicate changes across multiple comparisons, numerous analytical approaches have been developed and applied to large sets of data from microarray experiments. The algorithms used to classify genes and experiments fall into two general categories: supervised and unsupervised classifications. Unsupervised classification algorithms, also known as clustering algorithms, attempt to find patterns, trends, or clusters in a dataset, without prior knowledge of these patterns. The hypothesis is that groups of genes or EST, which may not be linked by the current state of knowledge of cellular function, associate biologically and may therefore support aspects of the observed phenotype. Software packages for unsupervised learning analysis, such as Cluster (http://rana.lbl.gov/) or GeneCluster (http://waldo.wi.mit.edu/MPR/software.html) can be freely downloaded from the internet. Cluster performs hierarchical clustering of correlated patterns of expression, in a similar way as evolutionary trees are created to identify families of related DNA sequences. GeneCluster, on the other hand, groups genes together according to predetermined criteria and numbers of patterns across all samples. The algorithms in clustering analysis use looser criteria than the stringent conditions for increase or decrease calls of the Affymetrix software. The differences of hybridization efficiency between all pairs of perfect and mismatch probes for each genes or EST are averaged ('averaged difference'). This single value is used to characterize the best estimate of expression level of a given gene or EST and is used with GeneCluster. For hierarchical clustering with Cluster, fold differences of gene expression levels between two experimental groups are used. Hierarchical clustering strategies have been applied to a fairly diverse set of experimental conditions to cluster genes with correlated expression patterns (Eisen et al., 1998; Tamayo et al., 1999).

On the other hand, supervised algorithms, such as Support Vector Machine (Brown et al., 2000, http://www.cs.columbia.edu/~bgrundy/svm/doc/svm.html), have not been applied as often to gene expression data. The goal of this type of analysis is to identify a 'signature' of the experimental outcome and to use this knowledge to predict a behavioral or physiological outcome from microarray data. Supervised clustering also exploits prior knowledge of gene function to identify unknown genes of similar function from expression data. It allows to predict functional roles for uncharacterized genes or ESTs, as well as 'physiological phenotypes' by recognizing hidden patterns of gene expression after being trained on a database of labeled experiments. Direct applications of this approach include identifying cellular responses to drug exposure in animal models and the ability to predict a therapeutic phenotype by looking at patterns of gene expression. For instance, by comparing the transcriptional effects of novel compounds to patterns induced by various antidepressant treatments, one may predict the likelihood of positive therapeutic outcome.

4. Conclusion

Although the current approaches to genetic manipulations have provided considerable insights into the contribution of several serotonin receptors to normal and pathological physiology (Lucas and Hen, 1995; Murphy et al., 1999), the analysis of genes with complex expression patterns, such as the 5-HT_{1A} receptor, required the development of more refined genetic interventions. In particular, the dissection of mechanisms of neuropsychiatric disorders and their therapeutic treatments will need an analysis of their molecular components and of their contributions to brain function in specific areas of the central nervous system, either during development or in the adult organism. Here, we have presented a combined experimental approach of tissue specific and conditional genetic manipulations, with large-scale genomic search for markers of altered function. These new generations of mutant mice are providing better models for mental disorders, such as depression or anxiety. In particular, these new models offer the ability to reproduce the variable intensity of neural network disturbances that occurs in human patients. Moreover, the inducible nature of the genetic manipulation allows us to investigate changes in function over time, by mimicking, for instance, pre-disease state, appearance of symptoms and recovery. Lastly, the monitoring of expression levels of thousands of genes and ESTs, in parallel to the genetic manipulations represent a powerful tool for identifying new gene products whose activity correlate with different physiological states. Changes in expression levels that are reliably detected by microarrays will be confirmed on a different platform (Northern blot, in situ hybridization or real-time PCR). The genes identified by these screens will then be submitted to further criteria to assess their relevance and potential value as new targets for pharmacological intervention.

While constitutive knockout studies will keep bringing fundamental information on the role of many genes, the combination of new genetic and genomic approaches represent considerable improvements over traditional approaches, in particular for studying complex genes. In our laboratory, the 5-HT_{1A} receptor has been used as a model system of gene product with distinct regional (separate brain regions) and temporal (developmental stages) functions. Accordingly, the increased anxiety phenotype of mice lacking the 5-HT_{1A} receptor is difficult to interpret from a mechanistic point of view, mostly due to a dual pre-

and post-synaptic localization of this receptor subtype within the serotonin system. By re-introducing the 5-HT_{1A} receptor in brain areas that are targeted by serotonin, such as hippocampus, cortex and septum, or in serotonergic neurons of the raphe nuclei, we can disentangle pre- and post-synaptic contributions of this receptor to the observed phenotype of the constitutive knockout mice. Furthermore, the inducible nature of the genetic manipulation will allow to accurately mimic chronic blockade of the receptor during critical time periods, such as development or maturation phases of particular brain regions and/or neural networks. By blocking the expression of the receptor in restricted periods of time, we circumvent the compensations that possibly occur during the constitutive deletion of the receptor throughout the lifespan of the animal. Importantly, by monitoring altered gene expression in combination with temporal and regional manipulations of the 5-HT_{1A} receptor, we can identify markers or downstream effectors of receptor function whose expression levels correlate between the phenotype in behavioral screens and the expression of the receptor in a particular brain region at a defined time.

Taken together, combining temporal and regional genetic manipulations with large-scale gene expression profile represent news levels of sophistication for investigational procedures, especially when applied to assess the role of complex genes in the brain.

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