BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 275–279 (1996)
ARTICLE NO. 1792

Identification of Hypothalamic Transcripts Upregulated by Antidepressants

Ma-Li Wong,[1] Pooja Khatri,[1] Julio Licinio, Anna Esposito, and Philip W. Gold

Clinical Neuroendocrinology Branch, National Institute of Mental Health, NIH, Bethesda, Maryland 20892-1284

Received November 1, 1996

Identification of quantitative changes in gene expression that occur in the brain after antidepressant treatment can yield novel molecular markers that may be useful in the diagnosis and treatment of major depression. Using a modification of the differential display polymerase chain reaction, we describe the isolation of two transcripts that are differentially expressed in the brain after an 8-week course of antidepressant administration, compared to saline-treated control animals. © 1996 Academic Press, Inc.

Melancholic depression is a syndromal illness characterized by physiological and psychological manifestations of hyperarousal. The physiologic symptoms of melancholic depression principally reflect abnormalities in hypothalamic centers regulating circadian rhythms, feeding, growth and reproduction, and pituitary-adrenal function, resulting in a phase advance of the rest activity cycle (early morning awakening), anorexia, hyposomatoropism, hypothalamic hypogonadism, and hypercortisolism. The cardinal psychological manifestation of melancholia is intense anxiety, particularly about the self, and hopelessness about the prospects for a worthless self for future gratification. Recent data indicate that a potentially important biological abnormality in melancholia is hypersecretion of hypothalamic corticotropin releasing hormone (CRH), an arousal producing neuropeptide which in experimental animals causes anxiety, decrease feeding, inhibition of the growth and reproductive axes, and pituitary-adrenal activation (1, 2). Our group has previously reported that a consistent effect of the chronic, but not acute administration of two prototypic categories of antidepressants [i.e. tricyclic antidepressants and serotonin selective reuptake inhibitors (SSRI)] is a significant decrease in the levels of parvocellular paraventricular (PVN) CRH mRNA levels (3). These effects were associated to an antidepressant augmentation of the mRNA levels of either type I or type II glucocorticoid receptors in the hippocampus, thought to play a principal role in restraining hypothalamic CRH neurons (3, 4). These antidepressants also consistently influence 5HT-2 receptors, alpha and beta-adrenergic receptors, gaba receptors, and a variety of other receptors found within the hypothalamus as well as in disparate extrahypothalamic areas (for review see ref. 5). It is currently unknown whether the capacity of these antidepressants to systematically influence these parameters is relevant to their efficacy in depression.

We report here a study of the effect of chronic administration of imipramine and fluoxetine to the rat at the level of gene transcription in hypothalamus. Chronic administration was utilized because of the well documented findings that antidepressants are generally effective after 3-5 weeks of administration rather than acutely (3). We used PCR-based differential display that allows the comparison of gene expression patterns between two cell (6) populations or between various organs (7, 8) for the study of genes that are regulated by two prototypic antidepressant drugs. Differential display has the potential of identifying a spectrum of molecular factors,

[1] Both authors contributed equally to this work.

0006-291X/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

known and unknown, that are differentially regulated in cells under various conditions. Expression is regulated at the level of transcription for most genes; therefore, the evaluation of gene transcripts that are similarly regulated by two prototypic antidepressant medications that have diverse pharmacological effects can become an important tool in the understanding of molecular factors involved in the response to antidepressant treatment and might also help us better understand the molecular neurobiology of major depression. Here we present two novel transcripts that are upregulated in the hypothalamus by prolonged administration of either tricyclic antidepressant or SSRI drugs.

MATERIALS AND METHODS

Animals. Studies were carried out in accordance with animal protocols approved by the National Institutes of Health. Male Sprague-Dawley rats (175-200 g, Harlan) were housed 3/cage at 24°C with lights on from 06.00 to 18.00 h. Rats were randomly separated into control and antidepressant groups and weighed weekly. Experimental animals received a 0.5 ml intraperitoneal (ip) injection daily for 8 weeks with 1.0 mg/kg of the SSRI fluoxetine hydrochloride (dissolved in 0.9% saline) and generously provided by Eli Lilly and Co. (Indianapolis, IN), or 5.0 mg of the tricyclic antidepressant imipramine (Sigma, St. Louis, MO) dissolved in 0.9% saline. Control animals consisted of rats injected with vehicle (0.9% saline).

Animals were sacrificed between 10:00 and 12:00h. Brains were removed, dissected, frozen and stored at −70°C until used. The block of hypothalamic tissue was 2 mm deep and was taken using the optic chiasm as the rostral limit and the mamillary bodies as caudal reference.

RNA isolation and RNA analysis. Isolation of total RNA obtained from hypothalamus was done using triZOL RNA reagent (Gibco BRL, Gaithesburg, MD) following the manufacturer's instructions. Total RNA was dissolved in RNase-free water, the amount of RNA estimated by spectrophotometery, and then treated with RNAse-free DNase (Promega, Madison, WI) 30 min at 37°C. After DNase treatment, RNA was clean-up using RNeasy columns (QIAGEN, Chatsworth, CA) following the manufacturer's protocol. Samples of total RNA (10 μg) were run in 2.2 M formaldehyde/1% agarose gels to confirm integrity of RNA samples.

Differential display. Differential mRNA Display analysis was carried out using RNAmap (GenHunter, Brookline, MA) and following the manufacturer's instructions. We studied total RNA obtained from hypothalamus of animals treated either with saline, imipramine, or fluoxetine (3 animals per condition). Total RNA (0.2 μg) was reversed transcribed in a 20 μl reaction and the degenerated oligo(dT) primer T12MN (where N stands for A, C, G, or T) as 3′ primers. The cDNAs were then amplified by PCR in the presence of a [^{35}S]dATP on a Perkin-Elmer 9600 thermal cycler. The reaction mixtures (20 μl) included arbitrary decanucleotides (RNAmap kit A and kit B, GenHunter) as 5′ primers and T12MN as 3′ primers. Parameters for PCR were as follows: denaturation at 94°C for 15 sec, annealing at 40°C for 2 min, and extension at 72°C for 30 sec for 40 cycles and then 72°C for 5 min. Radiolabeled PCR amplification products were analyzed by electrophoresis in denaturing 6% polyacrylamide gels. Duplicate reactions from identical samples of each RNA preparation were done and were run side-by-side; only those bands that were differentially expressed on both duplicate lanes were considered. Differentially upregulated bands were defined as those that were consistently present in at least 4 out of 6 antidepressant treated animals and absent from all the saline treated animals. Differentially downregulated bands were defined as those present only in the saline treated animals.

Portions of the polyacrylamide gel and 3MM Whatmann paper were cut, rehydrated in 150 μl H_2O for 30 minutes at 20°C and eluted by boiling for 30 minutes. After a 10 min spin, DNA fragments in the supernatant were precipitated with ethanol, re-dissolved in 10 μl of H_2O and reamplified using the same primer set and PCR conditions used for the differential display reactions.

Reverse Northern blot. Reverse Northern blot was performed as described by Mou *et al.* (9). Briefly, the RT reaction proceeded as above; however, oligo-dT rather than the anchored oligo-dT was used and the amount of input total RNA was increased to 5 μg. Radioactive DNA probe was produced from the cDNA reaction by random priming method (6). Random primers [pdN6, 4.2 μg/μl (Pharmacia Biotech, Piscataway, NJ)] and oligolabelling kit (Pharmacia) were used following the manufacture's instructions and using [^{32}P]-dCTP (Amersham, Arlington Heights, IL). The random primer reaction was allowed to proceed at 37°C for overnight, then stopped by the addition of 75 μl TE (10 mM tris, pH 7.8/1 mM EDTA). A Sephadex G50 spin column (Pharmacia) was used to separate incorporated from unicorporated radioactive nucleotide (6, 9). The alkali-denatured probe was used within a week of isolation.

DNA was blotted and fixed to Gene Screen Plus membrane (New England Nuclear, Boston, MA) according to the manufacturer's instructions. Membranes were cut to the size of the slot manifold and placed into the manifold and clamped after wetted with 0.4 M Tris-HCl, pH 7.5. DNAs were denatured, diluted and 250 to 500 ng DNA was fixed per slot. Pre-hybridization was done at 57°C for 4 hs (pre-hybridization buffer contained 10% dextran sulfate/1M NaCl/1% SDS); hybridization buffer contained 2×10^6 radioactive DNA probe per ml buffer and 50 μg per ml sheared herring sperm DNA and the membranes were hybridized overnight. Post-hybridization washes were done with 2 × SSC/1% SDS for 30 min. The wet membranes was exposed to X-OMAT film (Kodak) for 3-7 days.

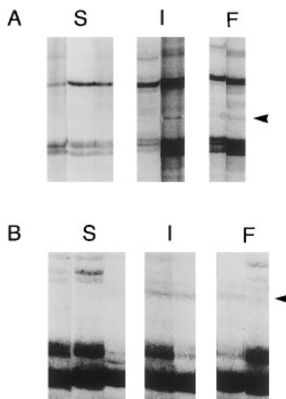

FIG. 1. Differential display comparing RNAs from saline- (S), imipramine- (I), or fluoxetine- (F) treated rats. Total RNA was extracted from hypothalamus from 2-month-treated animals. Autoradiograms of amplified $\alpha[^{35}S]$dATP-labeled PCR products (after electrophoresis in 6% polyacrylamide gels) are shown for two different primer combinations (A and B) that identified two distinct fragments (arrowheads) upregulated in the groups treated with antidepressant. Band #28 is shown in A and band #14 is shown in B.

RESULTS AND CONCLUSIONS

To identify potential genes transcriptionally regulated in the hypothalamus with prolonged antidepressant treatment, we compared differential mRNA display patterns of carefully dissected hypothalamus from rats treated with two prototypic antidepressant medications with those of saline-treated rats. We performed PCR amplifications with 40 primer combinations on all 9 samples and identified 28 PCR products, designated bands 1-28, that were differentially expressed between antidepressant and saline treated tissue. Fig. 1 shows PCR amplifications obtained with 2 separate primer combinations. Indicated are two representative PCR products (bands 14 and 28) that were present in the antidepressant treated samples (lanes 4-7) but not in the saline treated samples (lanes 1-3) in each of the two analyses.

To confirm the gene regulation patterns observed in the differential display study 23 out of 28 selected PCR bands were recovered, reamplified them, and blotted them into triplicate membranes. Each membrane was hybridized separately with radioactive DNA probe generated from RT reactions of hypothalamic tissue from rats treated with either one of the two prototypic antidepressant medications or from saline treated rats. Out of 23 recovered bands, 2 bands (14 and 28) shown in fig 1, were confirmed by the reverse northern blot (fig 2); and 11 bands could not be visualized in the reverse northern blot studies (data not shown). Such transcripts may not have been detected because their levels were below the sensitivity of the reverse northern blot analysis.

Our study demonstrates that the differential mRNA display technique can be adapted to identify mediators associated with complex CNS processes such as prolonged antidepressant treatment. Our analysis simultaneously compared a series of six animals treated with one of two prototypic antidepressant drugs, thus avoiding factors that might have been related to a single animal or single antidepressant drug. We performed each differential display reaction twice and ran the reactions side-by-side to reduce nonspecific (background) PCR signal interference, and we restricted the selection of cDNA bands for further study to those bands that were differentially expressed on both duplicate lanes.

Using 40 primer combinations we identified and recovered 23 differentially display cDNA bands that were reproducibly up-regulated in hypothalamus of antidepressant treated rats. For

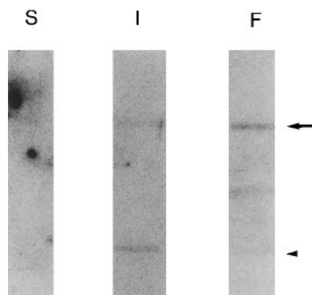

FIG. 2. Slot blot confirmation of antidepressant-mediated transcript induction for differential display bands shown in figure 1. Eluted DNA fragments were reamplified and equal amounts applied into triplicate membranes using a slot blot manifold. Autoradiograms of slot blot hybridized with radioactive dsDNA probes made from hypothalamic RNA of saline- (S), imipramine- (I), or fluoxetine- (F) treated rats areshown. Arrow corresponds to band shown in fig 1B and arrowhead to band shown in fig 1A.

2 of the 23 bands, this antidepressant treated regulation was reproduced on reverse northern blot analysis.

One of our goals in undertaking this study was to identify mediators that might be selective for or specific to prolonged antidepressant treatment: we identified two such transcripts. Having demonstrated a specific association between these genes and prolonged antidepressant injections, we can gain insight into their precise roles by isolating and analyzing their full-length cDNAs, identifying the specific cell types that express them (or their products), and studying their functions.

With the identification of these two candidate transcripts of mediators of prolonged antidepressant treatment, we demonstrate the potential of differential mRNA display analysis to provide insight into molecular factors associated with complex central nervous system processes. Antidepressant drugs are known to produce a variety of pharmacological effects on the brain biogenic amine systems, including inhibition of monoamine reuptake and alteration in 5-hydroxytryptophan $(5\text{-HT})_2$, dopamine, γ-aminobutiric acid (GABA), and α-and β-adrenergic receptors see (5) and to induce the resolution of the most consistent finding in patients with major melancholic depression, that is the activation of brain CRH and LC-NE systems (3, 4) thus, the power of this technique resides in its preservation of complex regulatory systems in the brain. *In vitro* systems investigating single cell types cannot reproduce the spectrum of CNS interaction present *in vivo* because they lack complexity and counterregulation. The differentially regulated transcripts we have identified are therefore likely to be of clinical relevance. In this regard it is important to recognize that major depression affects key functions of the hypothalamus, such as neuroendocrine regulation, food intake, and temperature control, and those functions return to normal after antidepressant treatment (1, 2). The differential display approach we used allows the identification of candidate factors that may be beyond the scope of established theories of therapeutic antidepressant actions and that might give us new information on hypothalamic dysfunction during major depressive disorder.

ACKNOWLEDGMENTS

We thank Sam Listwak (Clinical Neuroendocrinology Branch, National Institutes of Mental Health) for helping with sequencing gels and Margaret Levin (McConnel Lab, Department of Biological Sciences, Stanford University) for her helpful comments. This work was supported by a NARSAD (National Alliance for Research on Schizophrenia and Depression) Young Investigator Award to M.-L.W.

REFERENCES

1. Gold, P. W., Loriaux, D. L., Roy, A., Kling, M. A., Calabrese, J. R., Kellner, C. H., Nieman, L. K., Post, R. M., Pickar, D., Gallucci, W., and Chrousos, G. P. (1986) *N. Engl. J. Med.* **314,** 1329–1335.

2. Gold, P. W., Goodwin, F. K., and Chrousos, G. P. (1988) *N. Engl. J. Med.* **319,** 348–353, 413–420.
3. Brady, L. S., Whitfield, H. J., Fox, R. J., Gold, P. W., and Herkenham, M. (1991) *J. Clin. Invest.* **87,** 831–837.
4. Brady, L. S., Gold, P. W., Herkenham, M., Lynn, A. B., and Whitfield, H. J. (1992) *Brain Res.* **572,** 117–125.
5. Baker, G. B., and Greenshaw, A. J. (1989) *Cell. Mol. Neurobiol.* **9,** 1–44.
6. Liang, P., and Pardee, A. B. (1992) *Science* **257,** 967–971.
7. Welsh, J., Chada, K., Dalal, S. S., Cheng, R., Ralph, D., and McClelland, M. M. (1992) *Nucleic Acids Res.* **20,** 4965–4970.
8. Utans, U., Liang, P., Wyner, L. R., Karnovsky, M. J., and Russell, M. E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 6463–6467.
9. Mou, L., Miller, H., Li, J., Wang, E., and Chalifour, L. (1994) *Biochem. Biophys. Res. Commun.* **199,** 564–569.