Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7625–7628, October 1989
Neurobiology

Distribution of D_2 dopamine receptor mRNA in rat brain

(*in situ* hybridization/catecholamines/neuroanatomy)

JAMES H. MEADOR-WOODRUFF*†, ALFRED MANSOUR*, JAMES R. BUNZOW‡, HUBERT H. M. VAN TOL‡, STANLEY J. WATSON, JR.*, AND OLIVIER CIVELLI‡

*Mental Health Research Institute, Department of Psychiatry, University of Michigan, Ann Arbor, MI 48109-0720; and ‡Vollum Institute for Advanced Biomedical Research, The Oregon Health Sciences University, Portland, OR 97201

Communicated by Seymour S. Kety, July 13, 1989 (received for review April 8, 1989)

ABSTRACT **The distribution of mRNA coding for the D_2 dopamine receptor was studied in the rat brain by *in situ* hybridization. A cDNA probe corresponding to the putative third cytosolic loop and sixth and seventh transmembrane domains of the rat D_2 receptor was used to generate an ^{35}S-labeled riboprobe to hybridize to D_2 receptor mRNA. D_2 mRNA was found both in dopamine projection fields and in regions associated with dopamine-containing cell bodies, suggesting both postsynaptic and presynaptic autoreceptor localization. Highest concentrations of D_2 mRNA were found in neostriatum, olfactory tubercle, substantia nigra, ventral tegmental area, and the nucleus accumbens. This distribution is consistent with those reported with D_2 receptor autoradiography.**

The dopamine systems of the brain are well-studied, consisting of two major circuits and several local networks (1–3). The most extensive of the dopamine systems are the nigrostriatal circuit and the mesocorticolimbic system. These are of central importance in both regulation of motor control and modulation of emotional tone (4–6). Dysfunction of the nigrostriatal system, for example, has been implicated in a number of motor disturbances, especially Parkinson disease. Similarly, the mesocorticolimbic system has been postulated to be disturbed in some psychiatric conditions, most notably schizophrenia.

The receptor for dopamine has been resolved into two physiologically distinct subtypes, referred to as D_1 and D_2 (7). These subtypes were proposed to explain differences in coupling to adenylate cyclase; the D_1 receptor is considered to be stimulatory for this enzyme (8, 9), whereas the D_2 receptor is either inhibitory or unlinked (10, 11). It has become apparent that these two dopamine receptor subtypes are distinct, having unique pharmacological profiles, biochemical characteristics, neuroanatomical distributions, and apparent molecular weights (7). Thus far, the determination of the neuroanatomical distribution of the dopamine receptors has been limited to pharmacological binding studies involving receptor autoradiography. This technique has been extensively used to map the distributions of the D_1 and D_2 receptors in brain, specifically allowing the visualization and localization of the protein receptor (12–18).

The rat D_2 receptor has been cloned (19) and repeats all of the structural features common to the family of guanine nucleotide-binding regulatory protein (G protein)-coupled receptors, including the α_2 and β_2 adrenergic, muscarinic, rhodopsin, and serotonergic receptors. This family of receptors shares significant sequence similarity in the seven putative transmembrane domains and members of the family are highly variable in length and sequence in the region between the fifth and sixth hydrophobic domains, a region that has been associated with G-protein coupling (19, 20). With the advent of methods to determine the distributions of specific mRNAs and the cloning of the D_2 receptor, it is now possible to visualize mRNAs specific for the D_2 receptor protein in the brain, thus complementing previous studies designed to study the localization of D_2 binding sites.

In this study, we report on the distribution of D_2 receptor mRNAs in the rat brain, as determined by *in situ* hybridization. This technique allows certain key questions concerning this receptor to be addressed. For example, this technique localizes cell bodies, through visualization of mRNA instead of binding sites; it can thus be determined if the D_2 binding sites (12–18) are associated with local cell bodies. A second area that can be addressed is whether postsynaptic D_2 receptors are identical to or share considerable homology with presynaptic D_2 autoreceptors; if they are markedly dissimilar in primary peptide structure, a probe hybridizing to one would not recognize the other under high-stringency conditions. Finally, this technique will allow the direct study of regulatory questions in these systems; the ability to visualize and quantitate mRNAs in individual cells is a powerful tool for studying how other systems interact with the dopamine circuits.

METHODS

Animals and Tissue Preparation. Male Sprague–Dawley rats (250–300 g) were sacrificed by decapitation. The brains were rapidly removed and frozen in liquid isopentane (−30°C) for 30 sec. Frozen brains were sectioned in a Bright cryostat (20 μm) and thaw-mounted onto precleaned and polylysine-coated microscope slides. These sections were stored at −80°C until further preparation.

Riboprobe Production. A *Sac* I–*Bgl* II fragment of the rat D_2 cDNA previously reported by Bunzow *et al.* (19) was cloned into p64T7. This particular clone contains a 495-base-pair (bp) insert that codes for transmembrane domains VI and VII as well as the third cytosolic loop of the rat D_2 receptor. To produce antisense riboprobes, the 495-bp-containing clone was linearized with *Eco*RI; 1 ng of linearized plasmid was labeled with 250 μCi of uridine 5′-[α-[^{35}S]thio]triphosphate (UTP[^{35}S]; 1 Ci = 37 GBq) with T7 RNA polymerase. This reaction was in a final volume of 25 μl, containing 2 μl of dithiothreitol (0.1 M); 5 μl of the appropriate polymerase reaction buffer; 1 μl each of 10 mM ATP, 10 mM CTP, and 10 mM GTP; 1 μl of RNasin; and 1 μl of T7 RNA polymerase. After incubation at 37°C for 2 hr, labeled RNA was separated from unincorporated UTP[^{35}S] on a Sephadex G-50 column equilibrated with 0.1 M Tris·HCl

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: SN, substantia nigra; VTA, ventral tegmental area.
†To whom reprint requests should be addressed.

(pH 7.5) containing 12.5 mM EDTA, 0.15 M NaCl, and 0.2% $NaDodSO_4$ (21).

***In Situ* Hybridization.** Slides were removed directly from storage at −80°C and placed into 4% (vol/vol) formaldehyde for 60 min at room temperature. After three 5-min rinses in isotonic phosphate-buffered saline, sections were treated with proteinase K (1 μg/ml in 100 mM Tris·HCl, pH 8.0/50 mM EDTA) for 10 min at 37°C. Slides were then rinsed in water, rinsed in 0.1 M triethanolamine (pH 8.0), and treated with 0.1 M triethanolamine, pH 8.0/acetic anhydride, 400:1 (vol/vol), with stirring for 10 min. The sections were then rinsed in 2× SSC (300 mM NaCl/30 mM sodium citrate, pH 7.2) for 5 min, dehydrated through graded alcohols, and allowed to air-dry.

After this fixing and prehybridization, sections were treated with UTP[^{35}S]-labeled riboprobes. Probes were diluted in hybridization buffer [75% (vol/vol) formamide/10% (wt/vol) dextran sulfate/3× SSC/50 mM sodium phosphate, pH 7.4/1× Denhardt's solution/yeast tRNA (0.1 mg/ml)/sonicated denatured salmon sperm DNA (0.1 mg/ml)/10 mM dithiothreitol] to result in a final concentration of 2×10^6 dpm/30 μl (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). Diluted probe (30 μl and 50 μl) was applied to coronal and horizontal sections, respectively; a glass coverslip was applied and sealed into place with rubber cement. These slides were then incubated overnight at 55°C.

After hybridization, the cement and coverslip were removed, and the slides were rinsed in 2× SSC for 5 min. Slides were then treated with RNase A (200 μg/ml in 10 mM Tris·HCl, pH 8.0/0.5 M NaCl) for 30 min at 37°C. Subsequently, sections were rinsed in 2× SSC for 10 min, 1× SSC for 10 min, 0.5× SSC at 55°C (hybridization temperature) for 60 min, 0.5× SSC at room temperature for 10 min and then finally dehydrated in a graded alcohol series and air-dried. Sections at this point were either exposed to Kodak XAR-5 x-ray film for 1–3 days and subsequently developed or dipped in Kodak NTB2 emulsion and stored at 4°C for 6–17 days prior to development. Developed emulsion-dipped slides were processed through a graded series of alcohols and xylenes and mounted under a coverslip with Permount (21).

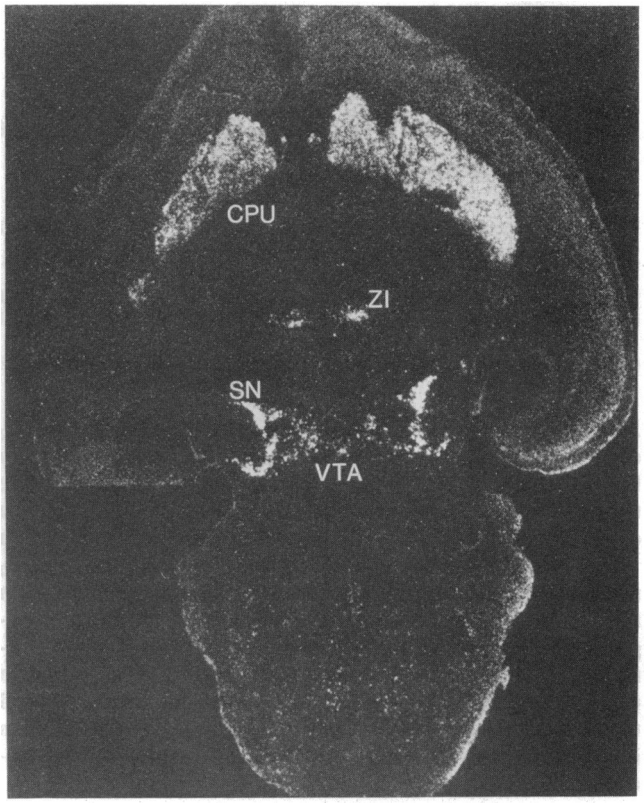

FIG. 1. Horizontal section of rat brain showing locations of D_2 receptor mRNA. The caudate-putamen (CPU) is intensely labeled, as are the major dopamine-containing cell groups that project to these structures, the SN and the VTA. Labeling of the zona incerta (ZI) is also apparent at this level.

RESULTS

D_2 receptor mRNA, detected by *in situ* hybridization, was found in all of the traditional dopamine terminal fields (Table 1 and Figs. 1–3). As can be seen from Figs. 1–3 and Table 1, the largest concentration was found in the neostriatum,

Table 1. D_2 mRNA in dopamine cell groups and projection areas

Cell group	Associated structures	D_2 mRNA	Associated projection area	D_2 mRNA
Mesencephalic				
A8			Caudate	+++
A9	SN	+++	Putamen	+++
A10	VTA	+++	Globus pallidus	+
			Nucleus accumbens	+++
			Olfactory tubercle	+++
			Prefrontal cortex	+
			Cingulate cortex	+
			Entorhinal cortex	++
			Piriform cortex	++
			Septum	+
			Olfactory bulb	++
			Amygdala	+
Diencephalic				
A11, A13 (incertohypothalamic)	Dorsal hypothalamus	+	Lateral septal nucleus	+
	Zona incerta	++	Hypothalamus	+
A12 (tuberoinfundibular)	Arcuate nucleus	(−)	Median eminence	(−)
			Neurointermediate lobe	+++
A14, A15	Medullary periventricular	(−)	Lateral septal nucleus	+
Other				
A16	Olfactory bulb	++	Local olfactory network	++
A17	Retina	ND		

+, Light hybridization; ++, moderate hybridization; +++, dense hybridization; −, nondetectable *in situ* signal; ND, not determined.

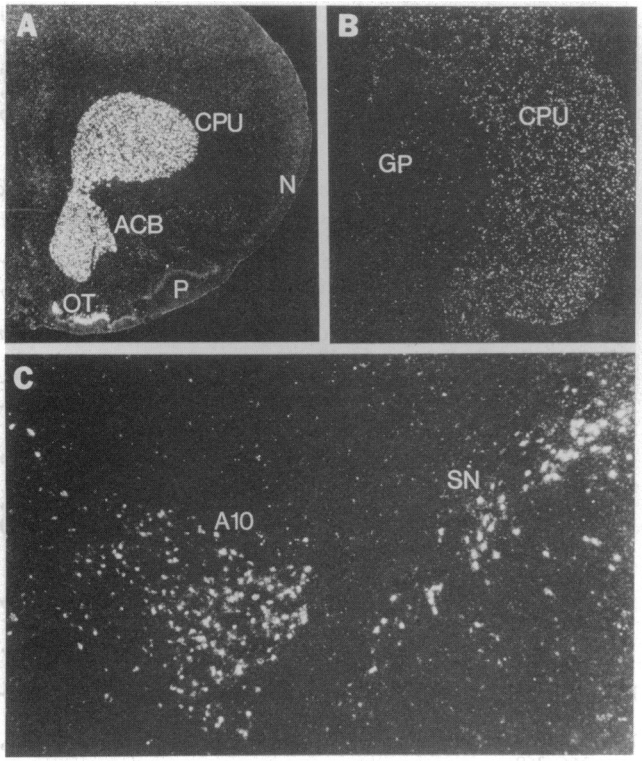

FIG. 2. D_2 receptor mRNA in the mesencephalic dopamine systems. (*A*) Rostral coronal section showing intense labeling of the caudate-putamen (CPU), nucleus accumbens (ACB), olfactory tubercle (OT), and piriform (P) cortex as well as faint labeling of neocortex (N). (*B*) Slightly more caudal than section in *A*, showing differential labeling in the caudate-putamen (CPU) and the globus pallidus (GP). (*C*) Caudal section showing labeling of the SN and the VTA (A10).

olfactory tubercle, and the nucleus accumbens. Somewhat lower amounts were detected in entorhinal and piriform cortex, as well as in olfactory bulb. D_2 receptor mRNA was also detected in other projection areas, including neocortex, cingulate gyrus, thalamus, hypothalamus, amygdala, and septum, as well as in the intermediate lobe of the pituitary. Although not considered by many as dopaminergic projection areas, low levels of D_2 receptor mRNA were also detected in hippocampus and in cerebellum.

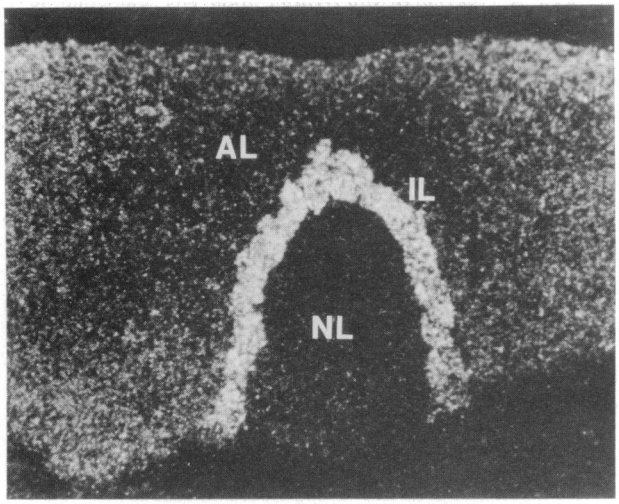

FIG. 3. Intense labeling in the intermediate lobe (IL) of the pituitary, the primary recipient of the tuberoinfundular (A12) dopamine projection. AL, anterior lobe; NL, neural lobe.

In addition to finding D_2 receptor mRNA in projection fields, this mRNA was also observed in some of the dopamine-containing cell groups that project to the previously listed areas. The highest levels were seen in the substantia nigra (SN) (especially the pars compacta) and the ventral tegmental area (VTA, A10). Clusters of D_2 mRNA-positive cells were identified in the region of A11, and the incerto-hypothalamic cell group (A13) was clearly labeled, especially the zona incerta (Fig. 1). The A16 cells in the olfactory bulb appeared to be labeled, but these cells remain local in the olfactory bulb, and the distinction between possible pre- and postsynaptic areas was difficult to make. The remaining dopaminergic cell groups were not found to contain D_2 mRNA. These negative groups include the tuberohypophyseal neurons located primarily in the arcuate nucleus (A12) and the medullary periventricular groups A14 and A15. The retinal A17 group was not studied.

To ensure the specificity of this labeling, several control studies were done (21). The hybridization signal observed in the caudate was able to withstand washes of up to 96°C in 0.5× SSC, in agreement with the predicted melting temperature (t_m) of the probe. Additionally, prehybridization of sections in the presence of RNase A obliterated any signal observed without this treatment. As a final control, the *Eco*RI–*Xho* II fragment of this 495-bp clone was subcloned into the *Eco*RI–*Bam*HI site of pGEM-4; this 205-bp fragment codes exclusively for the third cytosolic loop. A parallel mapping with this subclone produced results identical to those using the larger 495-bp clone.

DISCUSSION

The distribution of mRNA coding for the D_2 receptor has been determined in the rat brain by *in situ* hybridization; this technique has revealed D_2 receptor mRNA in some traditional dopamine-containing cell groups (1–3) and in associated major dopaminergic projections. It is conceivable that the probe that we have used in this study may be hybridizing to other receptors in addition to the D_2 receptor. We feel that this is unlikely, however, as the longest continuous region identical to any of the other G-protein-coupled receptors thus identified consists of a stretch of only 7 bp. The conditions and stringency used in this study would require hybridization of at least 40 bp to produce a signal after RNase treatment, strongly suggesting that we are indeed seeing only D_2 receptor mRNA. Northern blot analysis demonstrates a single band with this probe, again indicating probe specificity. Finally, the anatomical distribution reinforces these considerations, as it is consonant with the anatomy revealed by autoradiographic studies.

The largest of the dopamine systems in the brain consists of the two major midbrain groups, the SN and the VTA, and their rostral projections to numerous structures, including the neostriatum, limbic cortex, and other limbic regions such as the septum, olfactory tubercle, nucleus accumbens, amygdala, and piriform cortex. In substantial agreement with previous studies utilizing D_2 receptor autoradiography (12, 15–18), this system appeared to contain the highest levels of D_2 receptor mRNA, in both the cell bodies of origin, and in the projection fields. The remaining D_2 projection fields from cell groups other than A8–A10 were also identified and are in good agreement with earlier studies identifying D_2 binding by receptor autoradiography (12–18). Other structures, such as the cerebellum and hippocampus, were also found to contain D_2 mRNA; although these two tissues are often sources of false-positive signals in *in situ* hybridization, it appears that in this particular case, the observed labeling is specific. Northern blot analysis of these tissues results in a single D_2 mRNA band, identical in size to the corresponding band from

neostriatum. Additionally, D_2 receptor binding has been identified (12, 14) in these two tissues.

In addition to finding D_2 receptor mRNA in the traditional dopamine projection fields, of particular interest is that this mRNA was also identified in areas associated with dopamine-containing cell bodies (1). It is of note that only certain of these cell groups contained D_2 receptor mRNA; cells containing D_2 receptor mRNA were noticeably absent in other dopamine cell groups. It is likely that the D_2 receptor mRNA seen in these dopamine cell groups represents mRNA coding for presynaptic dopamine autoreceptors. We have preliminary data[§] indicating that unilateral 6-hydroxydopamine lesions of the medial forebrain bundle cause depletion of D_2 receptor mRNA in the ipsilateral SN and VTA and increased levels in the caudate, suggesting that the D_2 receptor mRNA visualized in SN and VTA expresses autoreceptors, whereas the mRNA expressed in the caudate probably codes for postsynaptic receptors.

Receptor binding and electrophysiological studies have produced conflicting results over the existence of presynaptic dopaminergic autoreceptors in particular dopamine-containing cell groups (22, 23). Although certain areas clearly contain D_2 receptor mRNA (A8–A10, A11, and A13), the negative findings in the other hypothalamic groups need to be interpreted with caution; these groups are small compared to the SN and VTA, and the inability to localize them may be related to technical limitations rather than lack of receptor mRNA. The current results do not rule out the possibility that the D_2 autoreceptors and postsynaptic D_2 receptors may be structurally different, although they clearly share significant sequence similarity in the region coding for the sixth and seventh transmembrane domains and the third cytosolic loop. In this regard, it should be noted that we have determined that there is only one D_2 receptor gene in the rat (D. K. Grandy, M. A. Marchionni, H. Makam, R. E. Stofko, M. Alfano, L. Frothingham, J. B. Fischer, K. J. Burke-Howie, J.R.B., A. C. Server and O.C., unpublished observation).

The D_2 receptors in the brain have been implicated in a variety of neurological and psychiatric conditions, including Parkinson disease and schizophrenia (4). The most consistent and widely held theory of the biological substrate of schizophrenia involves increased central D_2 receptor activity (5, 6). The ability to determine the location and levels of D_2 mRNA by *in situ* hybridization will allow the performance of preclinical regulatory studies and the examination of specific regions in postmortem schizophrenic brains to further explore the possibility of disturbances of the D_2 system in this illness.

[§]Mansour, A., Meador-Woodruff, J. H., Camp, D. M., Robinson, T. E., Bunzow, J., Van Tol, H., Civelli, O., Akil, H. & Watson, S. J., 20th Annual Meeting of the International Narcotic Research Conference, July 9–14, 1989, Ste.-Adele, PQ, Canada.

This work was supported by Grant DA02265 from the National Institute on Drug Abuse, Grant MH422251 from the National Institute of Mental Health, and grants from the Theophile Raphael Fund, and the Lucille P. Markey Charitable Trust to S.J.W. and Huda Akil, and Grant MH45614 from the National Institute of Mental Health to O.C.

1. Hokfelt, T., Johansson, O. & Goldstein, M. (1984) in *Handbook of Chemical Neuroanatomy*, eds. Bjorklund, A. & Hokfelt, T. (Elsevier, Amsterdam), Vol. 2, pp. 157–276.
2. Cooper, J. R., Bloom, F. E. & Roth, R. H. (1986) *The Biochemical Basis of Neuropharmacology* (Oxford Univ. Press, New York), pp. 259–314.
3. Nieuwenhuys, R. (1985) *Chemoarchitecture of the Brain* (Springer, Berlin), pp. 14–19.
4. Seeman, P., Bzowej, N. H., Guan, H. C., Bergeron, C., Reynolds, G. P., Bird, E. D., Riederer, P., Jellinger, K. & Tourtellotte, W. W. (1987) *Neuropsychopharmacology* **1**, 5–15.
5. Creese, I., Burt, D. R. & Snyder, S. H. (1976) *Science* **192**, 481–483.
6. Seeman, P., Ulpian, C., Bergeron, C., Riederer, P., Jellinger, K., Gabriel, E., Reynolds, G. P. & Tourtellotte, W. W. (1984) *Science* **225**, 728–731.
7. Clark, D. & White, F. J. (1987) *Synapse* **1**, 347–388.
8. Kebabian, J. W. (1978) *Adv. Biochem. Psychopharmacol.* **19**, 131–154.
9. Spano, P. F., Govini, S. & Trabucchi, M. (1978) *Adv. Biochem. Psychopharmacol.* **19**, 155–165.
10. DeCamilli, P., Macconi, D. & Spada, A. (1979) *Nature (London)* **278**, 252–255.
11. Stoof, J. C. & Kebabian, J. W. (1981) *Nature (London)* **294**, 366–368.
12. Boyson, S. J., McGonigle, P. & Molinoff, P. B. (1986) *J. Neurosci.* **6**, 3177–3188.
13. Dawson, T. M., Barone, P., Sidhu, A., Wamsley, J. K. & Chase, T. N. (1988) *Neuroscience* **26**, 83–100.
14. Dawson, T. M., Gehlert, D. R., McCabe, R. T., Barnett, A. & Wamsley, J. K. (1986) *J. Neurosci.* **6**, 2352–2365.
15. van der Weide, J., Camps, M., Horn, A. S. & Palacios, J. M. (1987) *Neurosci. Lett.* **83**, 259–263.
16. Altar, C. A., Wasley, A. M., Neale, R. F. & Stone, G. A. (1986) *Brain Res. Bull.* **16**, 517–525.
17. Richfield, E. K., Young, A. B. & Penney, J. B. (1987) *J. Comp. Neurol.* **262**, 446–463.
18. Dubois, A., Savasta, M., Curet, O. & Scatton, B. (1986) *Neuroscience* **19**, 125–137.
19. Bunzow, J. R., Van Tol, H. H. M., Grandy, D. K., Albert, P., Salon, J., Christie, M., Machida, C. A., Neve, K. A. & Civelli, O. (1988) *Nature (London)* **336**, 783–787.
20. Dohlman, H. G., Caron, M. G. & Lefkowitz, R. J. (1987) *Biochemistry* **26**, 2657–2664.
21. Watson, S. J., Sherman, T. G., Kelsey, J. E., Burke, S. & Akil, H. (1987) in *In Situ Hybridization*, eds. Valentino, K. L., Eberwine, J. H. & Barchas, J. D. (Oxford Univ. Press, New York), pp. 126–145.
22. Filloux, F., Liu, T. H., Hsu, C. Y., Hunt, M. A. & Wamsley, J. K. (1988) *Synapse* **2**, 521–531.
23. El Mestikawy, S., Glowinski, J. & Hamon, M. (1986) *J. Neurochem.* **46**, 12–22.