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# Internalization and Trafficking of Opioid Receptor Ligands in Rat Cortical Neurons

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**KEY WORDS** deltorphin; dermorphin; confocal microscopy; opiate receptor; fluorescent peptide; primary neuronal culture

**ABSTRACT** The binding, internalization, and trafficking of the fluorescently labeled opioid peptides Fluo-dermorphin and Fluo-deltorphan were quantitatively studied by confocal microscopy in primary cortical neurons in culture. Specific binding of these selective ligands to neurons naturally expressing mu ( $\mu$ ) and delta ( $\delta$ ) opioid receptors (OR), respectively, resulted in their internalization into neuronal somas and processes, as indicated by the persistence of fluorescent labeling following removal of cell surface binding by hypertonic acid wash. This internalization was receptor-specific, as the fluorescent signal was completely abolished when the cells were concomitantly incubated with the opioid receptor antagonist naloxone. It also was clathrin-dependent, as it was totally prevented by the endocytosis inhibitor phenylarsine oxide. Accordingly, internalized ligands were detected inside small, endosome-like vesicles. These labeled vesicles accumulated within nerve cell bodies between 5–30 min of incubation with the fluorescent ligands. This accumulation was abolished after treatment with the anti-tubular agent nocodazole, suggesting that it was due to a microtubule-dependent, retrograde transport of the internalized ligands from processes to the soma. By contrast, there was no change in the compartmentalization of internalized  $\mu$ OR or  $\delta$ OR, as assessed by immunocytochemistry, suggesting that the latter were recycled locally. The present results provide the first demonstration of receptor-mediated internalization of opioid peptides in cultured neurons. It is proposed that their retrograde transport into target cells might be involved in mediating some of the long-term, transcriptional effects of opioids. **Synapse 43:102–111, 2002.** © 2001 Wiley-Liss, Inc.

## INTRODUCTION

Peptides and alkaloids that activate opioid receptors influence a variety of processes, including pain, motor activity, and gastrointestinal motility (Vaccarino et al., 1999). Three opioid receptors (ORs) have been identified and cloned to date, termed mu ( $\mu$ ), delta ( $\delta$ ), and kappa ( $\kappa$ ) (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Thompson et al., 1993). While activation of each opioid receptor subtype elicits analgesia, available therapeutic drugs primarily act as agonists at the  $\mu$ OR. Accordingly,  $\mu$ OR agonists remain the first choice of therapy for severe and chronic pain despite their potential for the development of tolerance, dependence, and addiction.

Cell signaling following agonist stimulation of opioid receptors is tapered by receptor desensitization and downregulation, two events mediated by receptor uncoupling from G-proteins and internalization (Ferguson et al., 1998). It has been suggested that opioid receptor internalization may also contribute to the de-

velopment of tolerance and dependence (Whistler et al., 1999). Indeed, it has been shown that, whereas the endogenous opioids, enkephalins, and endomorphins, and the alkaloid etorphine, all induce OR endocytosis, morphine does not (Keith et al., 1996; McConalogue et al., 1999; Sternini et al., 1996; Trafton et al., 2000). Given that mice chronically treated with etorphine develop less physiological tolerance than do mice treated with equi-effective doses of morphine (Duttaroy and Yoburn, 1995), these results were taken to imply that internalization may be linked to the ability of opioid drugs to induce tolerance (Keith et al., 1998). This hypothesis was further validated by the report that

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methadone and morphine differ in their ability to induce both  $\mu$ OR internalization and addiction (Whistler et al., 1999) and by the observation that mice in which the gene for the adapter protein  $\beta$ -arrestin has been knocked out show a markedly decreased tolerance to morphine (Bohn et al., 2000).

Ligand-induced internalization has long been known to play important cellular functions in addition to receptor desensitization and downregulation. In particular, it has been linked to receptor resensitization allowing for functional recovery (Grady et al., 1995; Ferguson et al., 1998), as well as to regulation of gene transcription (Belcheva et al., 1996; Ventura et al., 1998; Ignatova et al., 1999; Souazé et al., 1997; Sarret et al., 1999). Most of our knowledge concerning the mechanisms of OR internalization emanates from studies *in vitro*, in immortal cells expressing either native or recombinant receptors (von Zastrow et al., 1993; Arden et al., 1995; Keith et al., 1996; Trapaidze et al., 1996; Gaudriault et al., 1997). Thus, studies from our laboratory have demonstrated that  $\mu$ OR and  $\delta$ OR, when coexpressed in the same cells, internalized through partly distinct endocytic pathways and were sorted via different endocytic vesicles (Gaudriault et al., 1997). Endocytosis of both  $\mu$ OR and  $\delta$ OR was shown to be clathrin-dependent, based on its sensitivity to a variety of clathrin disrupters (Keith et al., 1996; Trapaidze et al., 1996; McConalogue et al., 1999; Burford et al., 1998). There is now abundant evidence that internalization of  $\mu$ OR occurs in neurons as well as in epithelial cells, both *in vitro* (Sternini et al., 2000; Keith et al., 1998; McConalogue et al., 1999) and *in vivo* (Sternini et al., 1996; Eckersell et al., 1998; Trafton et al., 2000). Little is known, however, regarding  $\delta$ OR internalization in either central or peripheral neurons, although this receptor subtype has been shown to internalize in heterologous transfection systems (Zhang et al., 1999). Furthermore, there is no information available regarding the fate and trafficking of either  $\mu$ OR or  $\delta$ OR, or of that of opioid ligands, following their internalization in neurons.

In the present study we demonstrate receptor-specific internalization of selective opioid receptor ligands in cortical neurons in culture. In addition, we show that ligands and receptors undergo differential trafficking patterns following internalization.

## MATERIALS AND METHODS

### Cortical neuronal culture

Cerebral cortices were isolated from the brains of newborn Sprague Dawley rat pups (P1), washed with Hank's Balanced Salt Solution (Gibco-BRL, Grand Island, NY), and incubated in the presence of Trypsin-EDTA (Gibco-BRL) for 15 min at 37°C. After washing, the cells were mechanically separated by gentle trituration through fire-polished Pasteur pipettes of decreasing bore diameter. The cell suspension was fil-

tered through a 70- $\mu$ m sterile filter and cells were plated onto poly-L-lysine-coated coverslips at a density of  $2 \times 10^5$  cells/well. The growth medium was composed of DMEM (Gibco-BRL) supplemented with 20 mM KCl, 110 mg/L sodium pyruvate, 2 mM glutamine, 0.9% glucose, 0.1% penicillin and streptomycin (Gibco-BRL), 0.5% fungizone, 2% B27 (Gibco-BRL), and 1% fetal bovine serum (Harlan, IN). Neurons were routinely maintained in culture for 6–10 days (at which point they were fully differentiated) without any change of growth medium in an atmospheric condition of 5% CO<sub>2</sub> 95% air at 37°C.

### Binding of $\omega$ -Bodipy red-dermorphin and -deltorhin in primary cortical cultures

The fluorescent agonists  $\omega$ -Bodipy 576/589 [K7]DRM-I 5APA (Fluo-DRM) and  $\omega$ -Bodipy 576/589 DLT-I 5APA (Fluo-DLT) were synthesized and purified as described previously (Gaudriault et al., 1997). For monitoring the binding and internalization of these fluorescent ligands to primary cortical neurons, cells were preincubated for 10 min at 37°C in Earles buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 0.9 mM MgCl<sub>2</sub>, and 25 mM HEPES), supplemented with 0.8 mM 1,10 phenanthroline (Sigma, St. Louis, MO), 0.09% glucose, and 0.2% bovine serum albumin (BSA). They were then incubated with either Fluo-DRM or Fluo-DLT diluted in the same buffer for either 5 or 30 min at 37°C. At the end of the incubation, neurons were washed in Earles buffer containing 0.5 M NaCl and acetic acid (pH 4.0) to dissociate surface-bound ligand; they were then fixed with 4% paraformaldehyde (PFA), rinsed with ice-cold Earles buffer, and mounted onto glass microscope slides with Aquamount. Specificity of ligand binding and internalization was determined by co-incubation of ligands with 10  $\mu$ M naloxone, a non-selective OR antagonist.

In order to determine whether internalization of ligand-receptor complexes was mediated through clathrin-coated pits, neurons were incubated for 30 min in supplemented Earles buffer containing the fluorescent ligand in the presence or absence of 10  $\mu$ M phenylarsine oxide (PAO). In order to determine whether the intraneuronal trafficking of internalized ligand was microtubule-dependent, internalization assays were performed in the presence or absence of nocodazole, a microtubule assembly blocker. To this aim, neurons were preincubated for 30 or 60 min in equilibration buffer containing 10  $\mu$ M nocodazole prior to the binding of fluorescent ligands.

### Immunocytochemistry on cultured neurons

Cultured neurons were incubated, or not, with non-fluorescent DRM or DLT for 5 or 30 min prior to washing with 0.1 M PB, pH 7.4, and fixing with 4% PFA for 20–30 min at 37°C. Cells were further washed with 0.1

M PB followed by 0.1 M Tris-buffered saline (TBS), pH 7.4, and incubated in TBS containing 10% normal goat serum (NGS) and 0.1% Triton X-100 for 15 min at 37°C. They were then incubated overnight at 4°C either with a  $\delta$ OR antibody directed against residues 3–17 from the predicted amino acid sequence of the N-terminus of the mouse  $\delta$ OR (Chemicon, Temecula, CA), or with a  $\mu$ OR antibody directed against residues 384–398 from the predicted C-terminus of the rat  $\mu$ OR (Incstar Corp., Stillwater, MN). In order to assess the percentage of opioid receptor-immunoreactive neurons in our cultures, double-labeling experiments were performed by co-incubating the cells with either  $\delta$ OR or  $\mu$ OR antibodies and with an antibody directed against microtubule associated protein-2 (MAP-2), a neuronal marker (1:500; Boehringer Mannheim, Philadelphia, PA). Specificity of the OR antibodies was extensively tested by Western blotting and immunocytochemistry in non-transfected COS-7 cells and in COS-7 cells transiently transfected with cDNA encoding the  $\mu$ OR or  $\delta$ OR (Cahill et al., 2001; Lee, unpublished observations). Additionally, antibody specificity was demonstrated by the loss of immunoreactivity following co-incubation of either antibody with the appropriate antigenic peptide. All antibodies were diluted in 0.1 M TBS, containing 0.5% NGS and 0.1% Triton X-100, pH 7.4. After extensive washing with 0.1 M TBS, neurons were incubated with goat antirabbit-Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA), at a dilution of 1:200 in 0.1 M TBS, for 45–60 min at room temperature. For double-labeling experiments, this secondary antibody was used in conjunction with a FITC-tagged goat antimouse antibody (Jackson ImmunoResearch) at the same dilution. Finally, cells were washed and the coverslips were mounted onto glass microscope slides with Aquamount.

#### Confocal microscopy, image processing, image analysis, and statistical calculations

Neurons were examined under a Zeiss laser scanning microscope attached to an Axiovert 100 inverted microscope (Carl Zeiss Canada, Don Mills, Ontario, Canada). Single optical sections were acquired through a trans-nuclear plane. The corresponding resolution was 8 scans/frame for the bodipy-labeled neurons and 32 scans/frame for the immunolabeled neurons. Red dyes, including Texas Red, Fluo-DRM, and Fluo-DLT were imaged in the LUTS mode. For FITC, the RGB mode was utilized. The parameters used for image acquisition of Fluo-DRM and Fluo-DLT labeling were set constant across all experimental conditions. The same principle of preset constant parameters was applied to the image acquisition of  $\mu$ OR- and  $\delta$ OR-immunoreactivity, allowing for comparison and evaluation of changes between experimental conditions.

The proportion of neurons exhibiting Fluo-labeling was assessed in three experiments by counting in each

case the total number of neurons in approximately 50 fields at a magnification of 40 $\times$ . Neuronal identity was determined by phase-contrast to discriminate cortical neurons from glial cells. To determine the proportion of  $\mu$ OR- and  $\delta$ OR-immunoreactive neurons, a total of approximately 200 neurons per experiment ( $n = 3$ ) were identified by MAP-2 immunolabeling and the proportion of either  $\mu$ OR- and  $\delta$ OR-labeled neurons was visually determined.

To quantify the intracellular density of internalized Fluo-DRM or Fluo-DLT, confocal images were analyzed using Biocom's 200 Photometric System for Image Analysis software (v. 1.4) running on an IBM-compatible computer (Biocom, ZA Courtaboeuf, B.P., France). Cell area, areas covered by labeled fluorescent "hot spots," surface, and number of fluorescent "hot spots" were obtained for each cell. A total of 20–30 cells were imaged and analyzed per condition ( $n = 3$ –6 experiments). Total surface of labeled areas was expressed as a ratio of the total cell surface area, the result of which is termed hereafter *label occupancy*. Statistical analyses for these internalization assays were done using Kruskal-Wallis one-way ANOVA.

To assess intracellular  $\mu$ OR or  $\delta$ OR immunoreactivity levels, confocal images were converted to a gray-scale and the integrated density per unit cell area (in arbitrary units, AU) was subsequently calculated using NIH ScionImage software program (Scion Corp., Frederick, MD). Surface area of neuronal somata (defined by the presence of the nucleus) and processes as well as integrated fluorescent densities were measured and processed on an IBM-compatible computer using Photoshop v. 4.0.1 and Adobe Illustrator v. 7.0 (Adobe Systems, San Jose, CA). A total of 15–20 cells from three independent experiments were analyzed. Statistical analyses for the immunolabeling studies were done using two-sample *t*-test combined with Dunn-Sidak and Bonferroni-adjusted probabilities.

## RESULTS

### Internalization of Fluo-DRM and Fluo-DLT in rat cortical primary cultures

The fluorescent derivatives of [Lys7] dermorphin and deltorphin-I, Fluo-DRM and Fluo-DLT, were used in the present investigation. The specificity and affinity of these opioid ligands for  $\mu$ OR or  $\delta$ OR, respectively, have been extensively characterized in transfected COS-7 cells (Fluo-DRM: IC<sub>50</sub>( $\mu$ ) 0.6 nM; ( $\delta$ ) 10 nM; Fluo-DLT: IC<sub>50</sub>( $\delta$ ): 2 nM; ( $\mu$ ) 1400 nM) (Gaudriault et al., 1997). Analysis of rat cortical cultures incubated for 30 min with 10 nM Fluo-DRM revealed that  $6.34 \pm 0.50\%$  ( $n = 142$ ) of neurons in these cultures were labeled with Fluo-DRM. By confocal microscopy, fluorescent labeling was highly punctate and distributed throughout both soma and processes (Fig. 1A). When cells were washed with hypertonic acid buffer to strip off surface-bound ligand, fluorescent hot spots were still present,

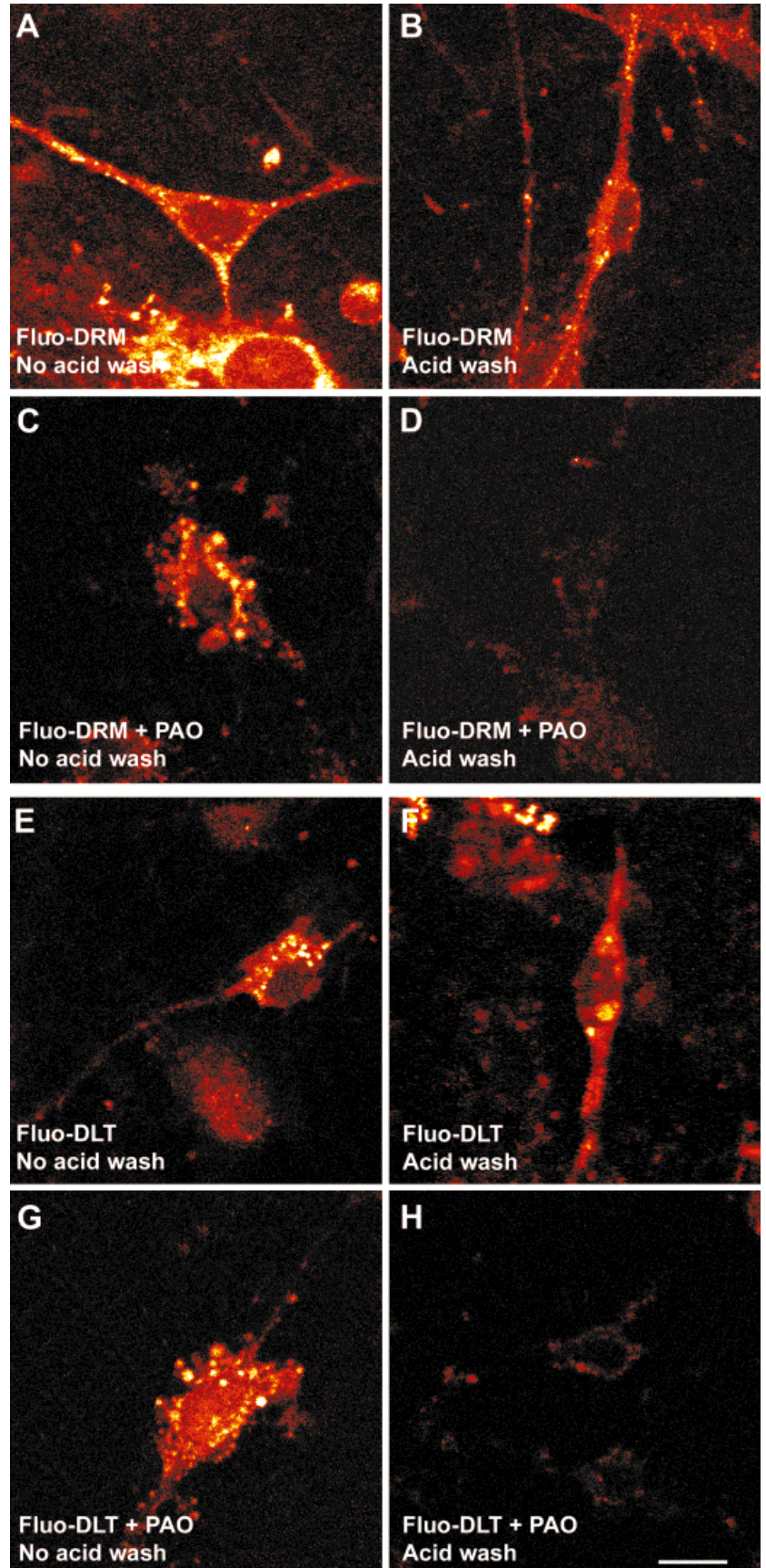


Fig. 1. Internalization of Fluo-DRM and Fluo-DLT in cortical neurons in culture. Neurons were incubated for 30 min at 37°C with 10  $\mu$ M Fluo-DRM (A–D) or Fluo-DLT (E–H) in the absence (A, B, E, F) or the presence (C, D, G, H) of 10  $\mu$ M phenylarsine oxide (PAO). A, C, E, G total binding; B, D, F, H residual binding after hypertonic acid wash. Confocal images were acquired through the nuclear plane at 8 scans per frame. Note the presence of intensely fluorescent endosome-like organelles in the cell soma and processes both prior to and after the removal of surface-bound ligand. Also note the disappearance of the fluorescent ligand in the presence of endocytosis inhibitor following hypertonic acid wash for both opioid receptor ligands. Scale bar = 10  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

albeit somewhat diminished in intensity, indicating that most of the label corresponded to internalized ligand (Fig. 1B). Moreover, binding and internalization of Fluo-DRM was receptor-specific in that it was no longer observed when neurons were co-incubated with 10  $\mu$ M naloxone (data not shown).

Similarly, 30-min incubation of cortical cultures with 10 nM Fluo-DLT at 37°C resulted in the selective fluorescent labeling of  $11.49 \pm 0.55\%$  ( $n = 348$ ) of neurons present in the culture. This labeling was specific in that it was totally abolished by the addition of 10  $\mu$ M naloxone to the incubation medium (data not shown). Examination of fluorescent labeling by confocal microscopy revealed diffuse punctate labeling located prominently in the soma and, to a lesser extent, inside neuronal processes (Fig. 1E). Here again, the labeling remained virtually unchanged following hypertonic acid wash, indicating that the fluorescent labeling corresponded to internalized ligand (Fig. 1F).

To determine whether Fluo-DRM and Fluo-DLT internalization was mediated via clathrin-coated pits, neurons were preincubated for 30 min at 37°C in an equilibration buffer containing the endocytosis inhibitor PAO (10  $\mu$ M). Binding of either ligand in the presence of PAO resulted in positive fluorescent labeling of cortical neurons (Fig. 1C,G). Both Fluo-DRM and Fluo-DLT labeling formed roundish plaques over the soma of PAO-treated cells; however, labeling along the processes was less prominent for both ligands. When neurons pretreated with PAO were subjected to hypertonic acid wash, neuronal fluorescent labeling was no longer detected, indicating that the fluorescent plaques corresponded to cell surface labeling (Fig. 1D,H).

#### Distribution of internalized fluo-ligands over time

In order to investigate ligand trafficking following internalization, Fluo-DRM and Fluo-DLT internalization was assessed after both 5 and 30 min of incubation. Quantification of the amount of internalized ligand at both time points was determined after acid-wash by measuring label occupancy, as described in Methods.

After 5 min of incubation with either Fluo-DRM or Fluo-DLT, a small number of acid wash-resistant fluorescent hot spots were observed throughout specifically labeled neurons (Fig. 2A,D). By 30 min there was a marked increase in the number of fluorescent hot spots over neuronal perikarya, but not at the level of processes (Fig. 2B,E). Quantification of confocal images indicated that the amount of internalized Fluo-DRM had doubled overall between 5 min ( $0.025 \pm 0.002$ ) and 30 min ( $0.050 \pm 0.008$ ) ( $P < 0.05$ ). Over the same period, the number of Fluo-DRM hot spots had also doubled (Table I). When somas and processes were analyzed separately, there was a significant increase with time in the amount of Fluo-DRM detected in the

cell soma (from  $0.011 \pm 0.001$  at 5 min to  $0.031 \pm 0.005$  at 30 min;  $P < 0.01$ ), but not at the level of processes ( $0.015 \pm 0.001$  at 5 min vs.  $0.016 \pm 0.003$  at 30 min;  $P = 0.566$ ) (Fig. 2C). In addition, there was a significant increase in the number, but not in the size, of hot spots in the somatic compartment (Table I). By contrast, no significant changes in either number or size of hot spots were seen in processes with time (Table I).

Quantification of internalized Fluo-DLT under the same conditions also showed a doubling of label occupancy of whole cells, from  $0.034 \pm 0.004$  after 5 min to  $0.060 \pm 0.009$  after 30 min ( $P < 0.05$ ). Over the same period, the number of Fluo-DLT hot spots also increased significantly (Table I). When somas and processes were analyzed separately, there was a significant increase in Fluo-DLT occupancy in the soma (from  $0.021 \pm 0.002$  at 5 min to  $0.049 \pm 0.007$  at 30 min;  $P < 0.01$ ; Fig. 2F), whereas no significant change was seen within processes between these time points ( $0.015 \pm 0.003$  vs.  $0.016 \pm 0.003$ ;  $P = 0.08$ ; Fig. 2F). In addition, there was a significant increase in the number, but not in the size, of hot spots in the somatic compartment (Table I). By contrast, no significant change in the number or size of hot spots was observed in processes with time (Table I).

To determine whether the increase in Fluo-DLT occupancy observed in the soma over time was due to retrograde transport of ligand internalized at the level of processes, additional experiments were performed in the presence of nocodazole, a microtubule assembly blocker. After 30 min of exposure to Fluo-DLT, there was a marked difference in the subcellular distribution of fluorescent hot spots between untreated and nocodazole-treated cells. In untreated controls, internalized Fluo-DLT was mostly observed over the soma, whereas in nocodazole-treated cells there was a widespread distribution of internalized Fluo-DLT over both soma and processes (Fig. 3A,B). Moreover, the intensity of Fluo-DLT labeling, if not grossly different as per whole neuron, differed significantly between soma and processes. Thus, Fluo-DLT occupancy of neuronal somas measured  $0.049 \pm 0.007$  in controls vs.  $0.019 \pm 0.003$  in nocodazole-treated neurons (Fig. 3C). Conversely, the label occupancy was markedly lower in the processes of untreated controls ( $0.016 \pm 0.003$ ) as compared to that in nocodazole-treated cells ( $0.029 \pm 0.006$ ;  $P < 0.05$ ) (Fig. 3C). Similar effects of nocodazole treatment were demonstrated for Fluo-DRM internalization; however, no quantitative analysis was performed as the size of the sample was too small.

#### Effect of ligand exposure on the distribution of OR immunoreactivity in cortical neurons in culture

Both  $\mu$ OR- and  $\delta$ OR-immunolabeled neurons exhibited immunofluorescence throughout their soma and processes (Fig. 4A,C). The proportion of  $\mu$ OR-immuno-

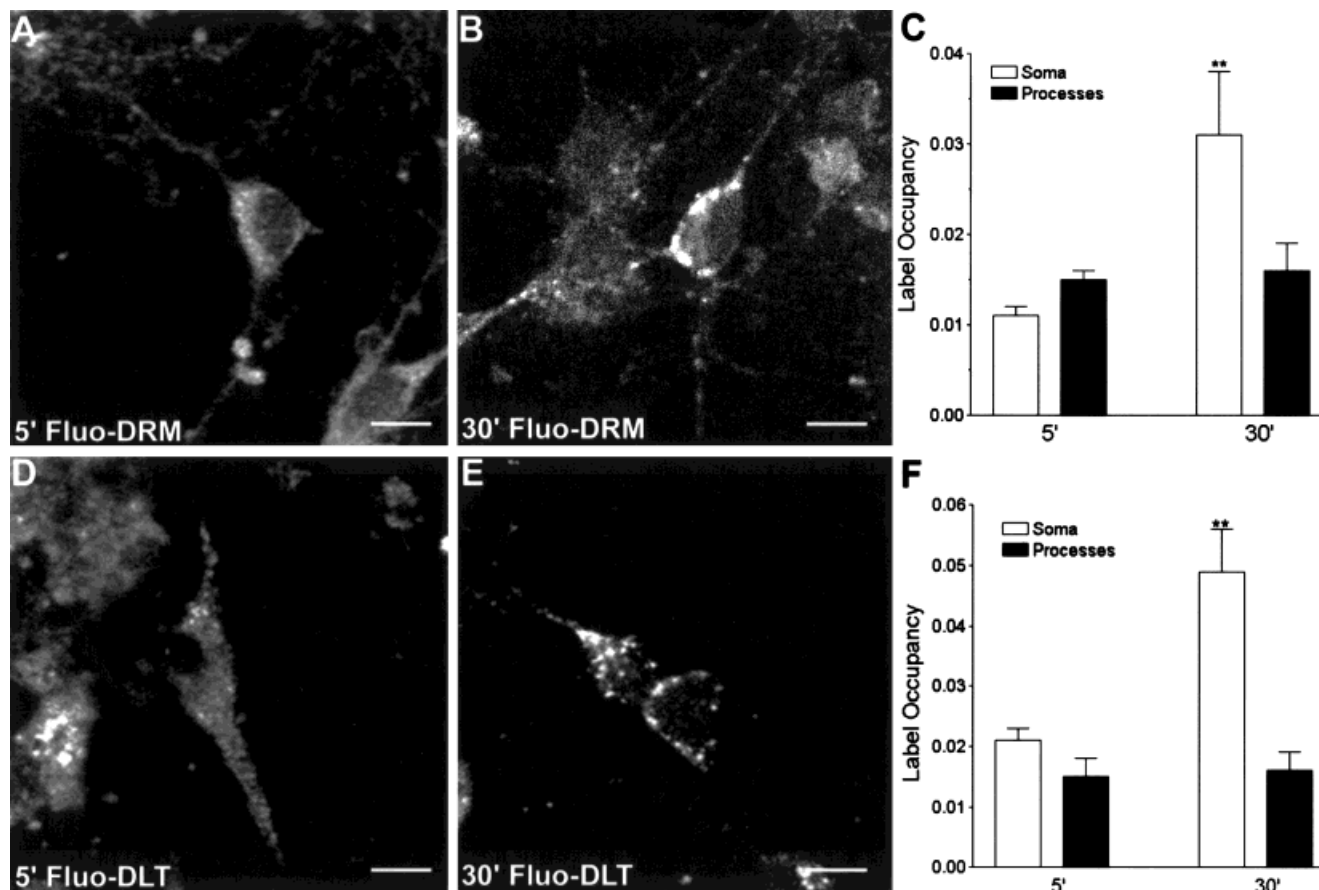


Fig. 2. Time-dependent changes in the compartmentalization of internalized Fluo-DRM and Fluo-DLT in cultured cortical neurons. Neurons were incubated for either 5 (A,D) or 30 (B,E) min at 37°C with 10 nM Fluo-DRM followed by hypertonic acid wash. A–C: Fluo-DRM labeling. Quantification of label occupancy (C) demonstrates a significant increase in the amount of Fluo-DRM accumulated in the soma between 5 and 30 ( $P < 0.01$ ) min, while there is no observable

change in the processes ( $P = 0.566$ ). D–F: Fluo-DLT labeling. Quantification of labeling occupancy (F) demonstrates a significant increase in Fluo-DLT accumulation in the soma between 5 and 30 min ( $P < 0.01$ ), while no observable changes occurred in the processes ( $P = 0.08$ ). Confocal optical sections were acquired at a resolution of 8 scans per frame with a Zeiss confocal laser scanning microscope. Scale bars = 10  $\mu\text{m}$ .

TABLE I. Intracellular compartmentalization of internalized ligands following 5 vs. 30-min incubations with Fluo-DRM and Fluo-DLT

	5 min			30 min		
	Processes	Soma	Total	Processes	Soma	Total
Fluo-DRM						
Size of fluorescent hot spots ( $\mu\text{m}^2$ ) <sup>a</sup>	0.103 $\pm$ 0.007	0.091 $\pm$ 0.007	—	0.084 $\pm$ 0.004	0.083 $\pm$ 0.008	—
Number of fluorescent hot spots <sup>b</sup>	9 $\pm$ 3	6 $\pm$ 2	15 $\pm$ 4	13 $\pm$ 4	18 $\pm$ 5 <sup>d</sup>	28 $\pm$ 10 <sup>d</sup>
Fluo-DLT						
Size of fluorescent hot spots ( $\mu\text{m}^2$ ) <sup>a</sup>	0.150 $\pm$ 0.012	0.154 $\pm$ 0.012	—	0.160 $\pm$ 0.018	0.173 $\pm$ 0.015	—
Number of fluorescent hot spots <sup>b</sup>	5 $\pm$ 2	7 $\pm$ 3	12 $\pm$ 5	6 $\pm$ 3	15 $\pm$ 6 <sup>c</sup>	21 $\pm$ 7 <sup>c</sup>

<sup>a</sup>Mean  $\pm$  SE; averaged over 20–30 cells/condition.

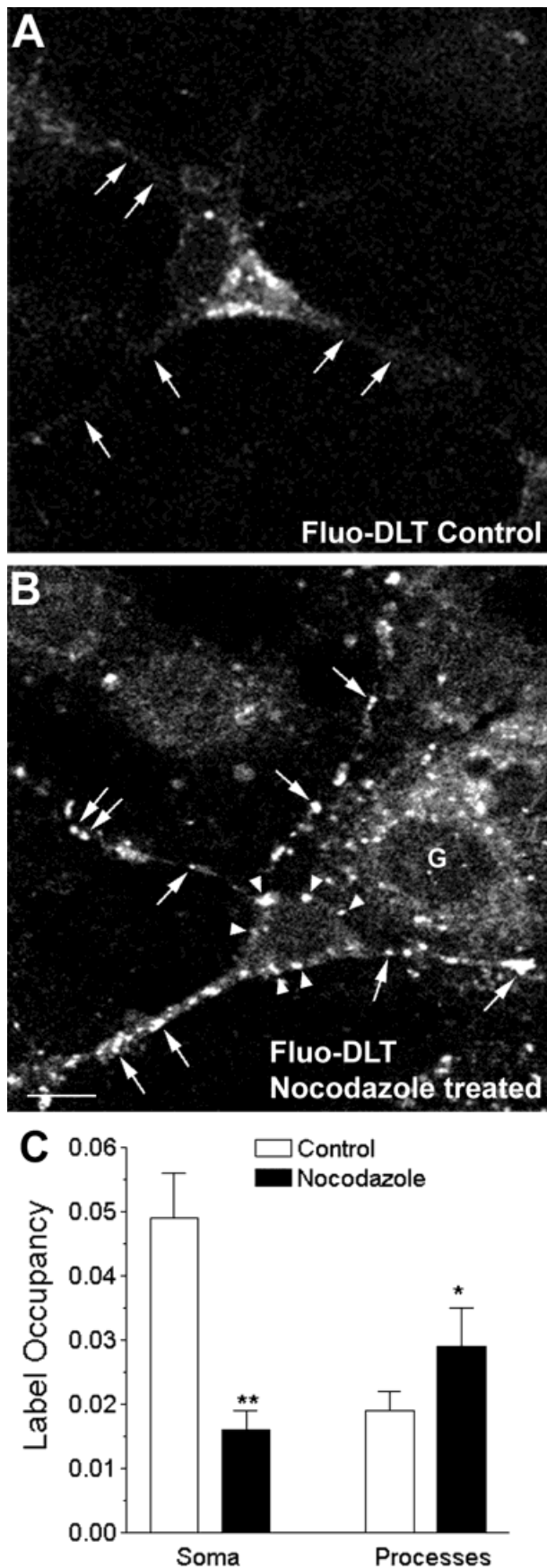
<sup>b</sup>Mean  $\pm$  SD/cell;  $n = 20$ –30 cells.

<sup>c</sup> $P_s < 0.01$  (within-group comparison between 5 and 30 min).

<sup>d</sup> $P_s = 0.001$  (within-group comparison between 5 and 30 min).

labeled cells, as determined in double-labeling experiments in which the whole neuronal population was labeled using the selective marker MAP-2 (Fig. 4B,D), was not significantly different from that found to bind Fluo-DRM ( $5.69 \pm 0.86\%$ ;  $P = 0.696$ ). Similarly, the proportion of cells that were immunopositive for  $\delta$ OR (Fig. 4A,C) was not significantly different from that labeled with Fluo-DLT ( $11.60 \pm 0.66\%$ ;  $P = 0.841$ ).

Preincubation of cortical neurons with nonfluorescent DRM or DLT for 5 or 30 min followed by immunostaining with  $\mu$ OR or  $\delta$ OR antibodies induced no significant time-dependent change in the cellular distribution of either  $\mu$ OR or  $\delta$ OR between somas and processes. Thus, the proportion of  $\mu$ OR immunolabeling associated with the soma was  $62.5 \pm 2.8\%$  of total at 5 min vs.  $65.0 \pm 1.0\%$  at 30 min ( $P = 0.420$ ), while



that associated with processes was  $37.5 \pm 2.8\%$  at 5 min vs.  $35.0 \pm 1.0\%$  at 30 min ( $P = 0.420$ ). Similarly,  $76.2 \pm 1.8\%$  of total  $\delta$ OR immunoreactivity was associated with the soma at 5 min vs.  $71.8 \pm 1.9\%$  at 30 min ( $P = 0.104$ ), while  $23.8 \pm 1.8\%$  was located within processes at 5 min vs.  $28.2 \pm 1.9\%$  at 30 min ( $P = 0.104$ ).

## DISCUSSION

The present study is the first to demonstrate receptor-mediated internalization of opioid ligands in central neurons. The results also show that this internalization is followed by retrograde transport of internalized ligands from processes to perikarya without comparable movement of internalized receptors, suggesting that the latter are recycled locally.

The present experiments were carried out on primary neuronal cultures from the cerebral cortex of newborn rats, as this region had been previously documented to abundantly express  $\mu$ OR and  $\delta$ OR both at birth (Nonomura et al., 1994; Kar and Quirion, 1995) and in adulthood (Mansour et al., 1995). Opioid ligand internalization was assessed by confocal microscopy following binding of selective fluorescent agonists at  $37^\circ\text{C}$  and acid-stripping of residual surface-bound ligand. As previously documented in COS-7 cells transiently transfected with cDNAs encoding  $\mu$ OR and  $\delta$ OR (Gaudriault et al., 1997), Fluo-DRM and Fluo-DLT bound with high affinity to neuronal  $\mu$ OR and  $\delta$ OR, respectively, as the opioid antagonist naloxone was able to competitively abolish the fluorescent cell labeling. Accordingly, the proportion of neurons found to specifically bind Fluo-DRM or Fluo-DLT was the same as that immunolabeled for either  $\mu$ OR or  $\delta$ OR. The extent to which these two populations overlap, that is, the proportion of neurons that express both  $\mu$ OR and  $\delta$ OR, remains to be determined.

Internalization of Fluo-DRM and Fluo-DLT is receptor-mediated, as it was prevented by the addition of a saturating concentration of naloxone. As in cells transiently transfected with either  $\mu$ OR or  $\delta$ OR cDNA (Gaudriault et al., 1997), the internalized label formed multiple, small intracytoplasmic fluorescent clusters that most likely corresponded to endosomes. Accordingly, internalization of both ligands was prevented by

Fig. 3. Internalized Fluo-DLT is targeted from processes to soma following internalization in cultured cortical neurons. Neurons were incubated for 30 min at  $37^\circ\text{C}$  with Fluo-DLT in the absence (A) or the presence (B) of  $10 \mu\text{M}$  nocodazole. C: Quantification of label occupancy in the soma and processes shows a significant decrease in fluorescent labeling in the soma of nocodazole-treated neurons compared to controls ( $P < 0.001$ ). Additionally, label occupancy of Fluo-DLT is markedly lower in the processes (arrows) of untreated controls compared to nocodazole-treated cells ( $P < 0.05$ ). Confocal optical sections were acquired at a resolution of 8 scans per frame with a Zeiss confocal laser scanning microscope. Arrowheads in B outline a Fluo-DLT-labeled nerve cell body. G denotes a glial cell. Scale bar =  $10 \mu\text{m}$ .



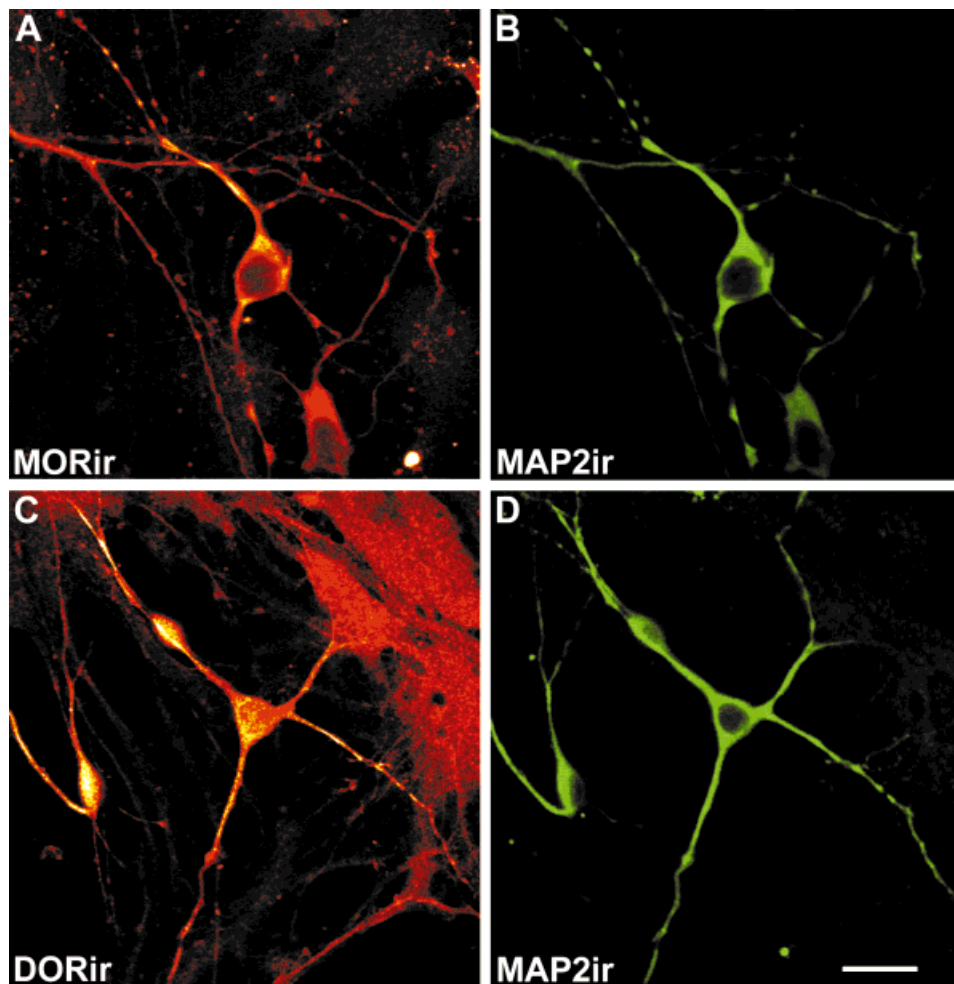


Fig. 4. Double immunocytochemical labeling of either  $\mu$ OR (A) or  $\delta$ OR (C) and of microtubule associated protein 2 (MAP2) (B,D) in primary cortical neurons in culture. Dissociated neurons were maintained in culture for 6–9 days and stained with either anti- $\mu$ OR or anti- $\delta$ OR antibody and MAP-2 followed by Texas Red-linked and FITC-linked secondary antibodies. Confocal optical sections were acquired at a resolution of 32 scans per frame with a Zeiss confocal laser scanning microscope. Scale bar = 10  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

the addition of the inhibitor of clathrin-mediated endocytosis, phenylarsine oxide. Ligand-induced, clathrin-mediated internalization of  $\mu$ OR had previously been documented in both neuronal (Keith et al., 1996; Sternini et al., 1996, 2000; Trafton et al., 2000) and non-neuronal cells (von Zastrow et al., 1993; Burford et al., 1998; Arden et al., 1995; Keith et al., 1996; Gaudriault et al., 1997; Whistler et al., 1999; Zhang et al., 1998).  $\delta$ OR had also been shown to internalize in a clathrin-mediated fashion, but only in transfected cell systems (Chu et al., 1997; Zhang et al., 1999) and in a neural cell line (Ko et al., 1999). The present study is the first, to our knowledge, to demonstrate agonist-induced internalization of  $\delta$ OR in primary neurons. This finding is consistent with the earlier demonstration that a variety of G-protein-coupled receptors internalize in neurons including, in addition to  $\mu$ OR, neurokinin 1 receptors (Mantyh et al., 1995a,b), muscarinic 2 receptors (Bernard et al., 1998), dopamine receptors (Dumartin et al., 1998), and somatostatin receptors (Boudin et al., 2000).

After 5 min of incubation with either Fluo-DRM or Fluo-DLT, fluorescent clusters were observed within perikarya and processes, indicating that ligand inter-

nalization proceeded at the level of both neuronal compartments. This observation is consistent with the reported distribution of both  $\mu$ OR and  $\delta$ OR over the entire neuronal surface (Cheng et al., 1997). The total amount of internalized fluorescence almost doubled between 5 and 30 min, confirming that the internalization process was time-dependent. This increase was entirely accounted for by accumulation of the fluorescent ligands in the somatic compartment, except in neurons treated with the microtubular disrupting agent, nocodazole, in which the amount of internalized ligand increased in processes, but decreased in the soma with time. These results suggest that Fluo-DLT and Fluo-DRM internalized at the level of neuronal processes are subsequently targeted to the somatic compartment via a microtubule-dependent transport mechanism. This interpretation is in keeping with the earlier demonstration of retrograde transport of another neuropeptide, neurotensin, following its internalization in central neurons. Indeed, neurotensin internalized at the level of the terminal field (Castel et al., 1992, 1994; Faure et al., 1995a) or dendritic arborization (Faure et al., 1995a) of midbrain dopaminergic or basal forebrain cholinergic neurons was found to pro-

gressively accumulate at the level of the perikarya of these cells. It was also shown that within neuronal perikarya, retrogradely transported material accumulated in a juxta-nuclear compartment (Faure et al., 1995a,b; Nouel et al., 1997). Although the nature of this compartment is still unclear, it was recently found to correspond to the trans-Golgi network (TGN) in transfected cells (Vandenbulcke et al., 2000). Further studies are required to determine if OR ligands are also targeted to the TGN in either wild-type cells or heterologous transfection systems.

Unlike fluorescent agonists, internalized ORs did not appear to be mobilized intracellularly across neuronal compartments, as there was no variation with time in the distribution of either  $\mu$ OR or  $\delta$ OR immunoreactivity between soma and processes. Admittedly, internalized receptors could be transported retrogradely, but the net levels of transported material might not be sufficiently in excess of those of reserve receptors to be detected by our immunocytochemical approach. It is also possible that the amount of receptors that are retrogradely transported is offset by that of receptors anterogradely migrating from the soma for replenishment of peripheral processes. It is more likely, however, that internalized receptors are merely recycled locally while dissociated ligand molecules are being transported towards the cell center. Indeed, both  $\mu$ OR and  $\delta$ OR have been shown to recycle efficiently in *in vitro* model systems (Zhang et al., 1998, 1999; Keith et al., 1998; Whistler et al., 1999). Furthermore, other G-protein-coupled receptors documented to recycle *in vitro* were found to do so at the level of neural processes *in vivo*. Thus, following nociception-induced internalization of NK-1 receptors within the dendritic arbors of nociceptive neurons of the rat dorsal horn, receptors internalized within endosomes were found to recycle back to dendritic plasma membranes without ever accumulating at the level of perikarya (Mantyh et al., 1995b). The lack of retrograde transport of ORs observed in the present study may therefore be common to many G-protein-coupled receptors.

Several lines of evidence have suggested that OR internalization may play a role in the development of tolerance and dependence. For one, opioid agonists have been shown to vary greatly in their capacity to induce receptor internalization and this capacity has been linked to the agonists' addictive properties (Zhang et al., 1998; Whistler et al., 1999). Thus, opioid agonists etorphine and morphine both activate  $\mu$ OR and  $\delta$ OR signal transduction, but only etorphine reportedly elicits, as do opioid peptides, internalization of these receptors (Keith et al., 1996; Cvejic and Devi, 1997; Sternini et al., 1996). More recent studies have reported a marked decrease in tolerance to morphine in mice in which the gene for  $\beta$ -arrestin, an adapter protein required for internalization, had been knocked out (Bohn et al., 2000). These results suggest that the develop-

ment of tolerance and dependence may be linked to receptor internalization and subsequent desensitization. The present demonstration that opioid ligands are also internalized and translocated from the processes to the cell body should perhaps be considered in the search for possible links between receptor internalization and the development of tolerance and/or dependence. Indeed, it has been previously proposed that retrograde transport of internalized neuropeptides may induce, perhaps through interaction of the ligand with intracellular receptors (Boudin et al., 2000), long-term transductional effects (Jans, 1994; Laduron, 1994). This type of genomic effect could well be involved in the development of behaviors such as tolerance and/or dependence.

In conclusion, this study demonstrates internalization of opioid peptides in neurons. We also show that  $\delta$ OR, as previously documented for  $\mu$ OR, internalize in neurons in a time-dependent and clathrin-mediated fashion, with kinetics comparable to those reported previously in nonneuronal cells. Finally, we show that following internalization internalized ligands dissociate from their receptors and are transported towards the center of the cell, via a microtubule-dependent mechanism, to the neuronal soma, whereas internalized receptors appear to be recycled locally.

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