Stoichiometry of a Ligand-gated Ion Channel Determined by Fluorescence Energy Transfer*

(Received for publication, August 10, 1998, and in revised form, December 28, 1998)

Sophie J. Farrar, Paul J. Whiting, Timothy P. Bonnert, and Ruth M. McKernan‡

From the Department of Biochemistry and Molecular Biology, Merck Sharp and Dohme Research Laboratories, Terlings Park, Eastwick Road, Harlow, Essex CM20 2QR, United Kingdom

We have developed a method to determine the stoichiometry of subunits within an oligomeric cell surface receptor using fluorescently tagged antibodies to the individual subunits and measuring energy transfer between them. Anti-c-Myc monoclonal antibody (mAb 9E10) derivatized with a fluorophore (europium cryptate, EuK) was used to individually label c-Myc-tagged α1-, β2-, or γ2-subunits of the hetero-oligomeric γ-aminobutyric acid (GABA_A) receptor in intact cells. The maximal fluorescent signal derived from the α1(c-Myc)β2γ2 and the α1β2(c-Myc)γ2 receptors was twice that obtained with α1β2γ1(c-Myc), suggesting that there are 2× α, 2× β, and 1× γ-subunits in a receptor monomer. This observation was extended using fluorescence energy transfer. Receptors were half-maximally saturated with EuK-anti-c-Myc mAb, and the remaining α1(c-Myc) subunits were labeled with excess anti-c-Myc mAb derivatized with the fluorescence energy acceptor, XL665. On exposure to laser light, energy transfer from EuK to XL665 occurred with 9-E10 derivatized with a fluorophore (europium cryptate; MEM, minimum essential medium; FCS, fetal calf serum) and the fluorescence energy acceptor, XL-665. Europium, encaged by cryptate, emits a strong long lived fluorescent signal at 620 nm when illuminated with light at 337 nm from a nitrogen laser, which can be time-resolved from short lived background fluorescence. In addition to being used as a single label, analogous to a radiolabel for example, EuK also serves as an energy donor. The fluorescent signal produced by EuK can be transferred to an acceptor molecule if it is in close enough proximity. The recipient molecule for this fluorescent resonance energy transfer is a modified allophycocyanine, XL665, which fluoresces at 665 nm (for review of the homogeneous time-resolved fluorescence technology see Mathis (1)). The transfer of energy from europium cryptate-labeled c-Myc antibodies to XL-665-labeled antibodies is therefore indicative of the two antibodies being in close proximity. Because the energy transfer is 50% at a distance of 9.5 nm (1), this would require that the antibodies are very closely associated, for example in the same macromolecular complex.

We used EuK-labeled c-Myc mAbs to quantify the number of subunits present on intact cells expressing α1β2γ2 subunits where each of the subunits was epitope-tagged with c-Myc and compared this with the number of receptors present using conventional radioligand binding. Fluorescence energy transfer was then used to confirm the stoichiometry of the receptor as (α1β2γ2)2.

Experimental Procedures

[methyl-3H]Ro15-1788 (87.0 Ci/mmol) was from NEN Life Science Products, Hertfordshire, United Kingdom. Flunitrazepam, GABA, and fetal calf serum were from Sigma. Minimal essential medium (MEM) was from Life Technologies, Inc.

Construction of c-Myc Epitope-tagged GABA_A Receptor Subunits and...

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 011-44-1279-440426; Fax: 011-44-1279-440390; E-mail: Ruth_McKernan@Merck.com.

The abbreviations used are: GABA_A, γ-aminobutyric acid, type A; GABA, γ-aminobutyric acid; mAb, monoclonal antibody; EuK, europium cryptate; MEM, minimum essential medium; FCS, fetal calf serum.
Stoichiometry of the \( \alpha_1\beta_2\gamma_2 \) Receptor

Transient Transfections—The GABA\(_\alpha\) receptor \( \alpha_1\)-subunit was epitope-tagged by site-directed mutagenesis using methods described previously (13). The epitope sequence (EQKLISEEDL) was inserted between Glu\(^{34}\) and Leu\(^{35}\) (these two residues becoming the first and last amino acid of the epitope tag), just C-terminal to the putative signal peptide cleavage site. The \( \beta_2 \) and \( \gamma_2 \) GABA\(_\alpha\) receptor subunits were epitope-tagged using a modified version of the pcDNA1Amp eukaryotic expression vector (pcDNA1AmpSignalMyc). This vector was constructed from the \( \alpha_1 \) c-Myc cDNA described above and contains the 5’-untranslated region of bovine GABA\(_\alpha\) \( \alpha_1\)-subunit (GenBank accession no. X05717), the signal peptide and six amino acids of the mature polypeptide of the subunit of interest can be inserted. The human \( \beta_2 \)-subunit c-Myc construct contains amino acids Glu\(^{34}\)–Asn\(^{474}\) of \( \beta_2 \), and the human \( \gamma_2 \) subunit c-Myc construct contains amino acids Tyr\(^{106}\)–Leu\(^{464}\). All constructs were confirmed by DNA sequencing. Constructs were prepared for transfection by CsCl centrifugation. Transient transfection into HEK293 cells was performed exactly as described previously in detail using a 1:1:1 ratio of \( \alpha_1 \), \( \beta_2 \), and \( \gamma_2 \) cDNAs (12, 15).

Radioligand Binding—The benzodiazepine site of the GABA receptor was labeled by the antagonist \[^{3}H\]Ro15-1788, a radioligand frequently used to quantify receptors, because only fully assembled receptor heterotrimers bind this ligand (7, 12, 16). Nonspecific binding was determined using 100 \( \mu \)M flunitrazepam. Binding to the GABA receptor was carried out at 3 \( \mu \)M antibody, and following 1 h of incubation with antibody, cells were washed twice before fluorescence at 620 nm was measured. A, binding to cells expressing the \( \alpha_1\) c-Myc/\( \beta_2\) subunit antisera bound to protein A-Sepharose (7). 50 \( \mu \)M of polyclonal antisera were incubated with 50 \( \mu \)M of packed protein A beads in a total volume of 1 ml of Tris-buffered saline for 1 h at room temperature. Receptors were solubilized from the cells using a deoxycholate/Triton buffer (1% Triton X-100, 0.5% deoxycholate, 0.1 mM KCl, 5 mM MgCl\(_2\), 1 mM phenylmethylsulfonyl fluoride, 100 mM Tris-HCl, pH 8.2) by mixing cell membranes for 1 h at 4 °C with a detergent buffer at a protein concentration of 1 mg/ml.

Aliquots (500 \( \mu \)l) of detergent-solubilized cell membranes were incubated with antibodies immobilized on protein A beads overnight at 4 °C. The receptor immobilized on protein A beads was washed three times with Tritis-buffered saline/0.1% Tween 20 by centrifugation and resuspension and was finally resuspended in 0.5 ml of Tris-buffered saline, and 10–50-\( \mu \)l aliquots of packed beads were used for \[^{3}H\]Ro15-1788 binding.

Generation of Europium Cryptate and XL665-labeled Antiserum—The monoclonal anti-c-Myc mAb, 9-E10 (ATCC no. CRL-1729, Ref. 14) was used for these experiments. An antibody was purified from hybridoma supernatant by Cymbus Biotech, UK, and this was then derivatized with either the fluorophore, europium cryptate (at an average ratio of 9 molecules of europium cryptate per antibody molecule), or with the fluorescence acceptor, XL665 (at a ratio of 1 molecule of XL-665 per antibody molecule), by Cymbio International, Marcoule, France. Labeling of c-Myc mAb with EuK at this ratio gave 418 counts of fluorescence/signal of antibody.

Binding of Europium Cryptate-labeled mAbs—Intact cells, transiently transfected with GABA\(_\alpha\) receptors with c-Myc-tagged subunits, were harvested by scraping, washed once by centrifugation (20 s at 1,000 rpm in a bench top Eppendorf Microcentrifuge), and resuspended in MEM + 5% FCS. \[^{3}H\]Ro15-1788 binding (1.8 nm) was carried out to determine receptor density on intact cells. Cells containing the equivalent of 100 fmol of receptor were incubated with various concentrations...
of europium cryptate-derivatized mAb (0.3–30 nM) in MEM + 5% FCS at room temperature, in a volume of 1 ml for 1 h on a rotamix wheel. After 3 × 1-ml washes in MEM + 5% FCS, the cells were resuspended in 190 μl of MEM + 5% FCS, and the fluorescence signal derived from europium-cryptate-labeled c-Myc mAbs was quantified in a 96-well low fluorescence microplate reader, following the addition of 10 μl of 1 M KF (final concentration, 50 mM KF). In preliminary experiments the inclusion of 5% FCS was found to improve the viability of the cells, and KF was included to stabilize europium cryptate and prevent oxidation of unliganded cryptate.

Energy Transfer to XL665-labeled Antibodies—XL665-derivatized mAbs were added in excess (10 nM) to each well, and the plate was read over a 24-h time period. Energy transfer was optimal after the receptor had been incubated with c-Myc-XL665 for 1 h, and the signal was stable for up to 18 h afterward.

RESULTS

Each of the α1-, β2-, and γ2-subunits was engineered to express a c-Myc epitope tag at the N terminus, and cells expressing c-Myc-tagged α-, β-, or γ-subunits or untagged receptors were generated by transient transfection. GABA_A receptors were measured using two ligands, [3H]muscimol, a radioligand for the GABA_A binding site, and [3H]Ro15-1788, a radioligand for the benzodiazepine binding site, which binds only to fully assembled receptors that contain an α-1, β-, and γ-subunit (7, 9).

Homogeneity of Expressed Receptors—In preliminary experiments it was important to confirm that a homogeneous population of fully assembled receptors was being expressed in the cells. This was done in two ways. First the number of binding sites for [3H]muscimol and [3H]Ro15-1788 was compared. Most evidence supports the presence of two [3H]muscimol binding sites and one [3H]Ro15-1788 binding site in a GABA_A receptor monomer in receptors immunoprecipitated from rat brain (7, 22). Therefore, if the majority of receptors is correctly assembled in cell lines, the ratio of B_max values for [3H]muscimol binding:[3H]Ro15-1788 binding should be 2:1. It is possible that receptors could be expressed that are composed of α- and β-subunits only, but these would not bind [3H]Ro15-1788, and the ratio would therefore be higher. Saturation analysis of [3H]muscimol and [3H]Ro15-1788 binding was carried out in cells expressing the untagged α1-, β2-, and γ2-subunits. Maximal binding of [3H]muscimol was 2067 ± 98 fmol/mg of protein with a K_d of 5.6 ± 0.7 nM (n = 3). Maximal binding of [3H]Ro15-1788 was 1226 ± 21 fmol/mg of protein with a K_d of 0.7 ± 0.05 nM (n = 3). The ratio of the GABA_A:benzodiazepine sites was 1.74 ± 0.11, which is in agreement with the expression of αβγ heterotrimeric receptors and no significant expression of receptors that contain only αβ-subunits.

Second, the ability of antibodies raised against the α1-, β2-, and γ2-subunit to immunoprecipitate all the [3H]Ro15-1788 binding sites from a solubilized cell membrane preparation was compared. The majority of receptors could be immunoprecipitated with antibodies raised against the α1-, β-, or γ2-subunits (α1, 74 ± 3%; β, 81 ± 7%; and γ2, 90 ± 4% of [3H]Ro15-1788 binding sites, n = 3). This confirms that the receptors that bind [3H]Ro15-1788 contain at least one α1, one β-, and one γ-subunit.

Expression of c-Myc-tagged GABA_A Receptors—The number of c-Myc-tagged subunits was quantified by labeling with anti-c-Myc mAb derivatized with europium cryptate, as illustrated in Fig. 1 using the subunit arrangement proposed by Tretter et al. (11) as a model).

The presence of a c-Myc tag on the N terminus of the GABA_A receptor subunits did not affect the expression of the receptor. The number of binding sites present on cells was unaffected by the addition of the epitope tag, and saturation analysis of [3H]Ro15-1788 binding showed that there was no major difference in the affinity of receptors for ligand (K_d) as is follows: α1β2γ2 = 1.3 nM; α1(c-Myc)β2γ2 = 1.1 nM; α1β2(c-Myc)γ2 = 1.4 nM; α1β2γ2(c-Myc) = 1.3 nM, n = 1). The density of receptors was unaffected by the expression of subunits with a c-Myc epitope tag, being 1.2–3.1 pmol/mg of protein. This is in agreement with previous studies where GABA_A receptors were tagged with c-Myc and FLAG epitopes without compromising binding, function, or modulation of the GABA_A receptor (12).

Labeling of GABA_A Receptors with EuK-c-Myc Monoclonal Antibodies—Preliminary experiments were carried out to determine the optimal conditions for the binding of EuK-labeled c-Myc mAbs. As shown in Fig. 2B, the fluorescence signal emitted by EuK-c-Myc at 620 nm was linear with receptor concentration. Therefore the binding of EuK-labeled c-Myc to the receptor was linear up to at least 100 fmol of receptor/ml. Background fluorescence from the plate and other reagents

FIG. 3. Saturation binding of EuK-c-Myc to intact cells expressing α1(c-Myc)β2γ2, α1β2(c-Myc)γ2, and α1β2γ2(c-Myc). Data shown are specific fluorescence counts bound after subtraction of nonspecific binding to cells expressing α1β2γ2. Data shown are from one experiment that was repeated three times with similar results. Data were fitted using Excelfit, and the binding parameters obtained were: B_max = 515 fluorescence counts/fmol of receptors and K_d = 6.2 nM for α1(c-Myc)β2γ2 (open circles); B_max = 602 fluorescence counts/fmol of receptors, K_d = 6.8 nM for α1β2(c-Myc)γ2 (closed circles); and B_max = 169 fluorescence counts/fmol, K_d = 4.4 nM for α1β2γ2(c-Myc) (open squares). For calculation of fluorescence counts/fmol of receptors, a receptor was quantified using [3H]Ro15-1788 binding at 1.8 nM.
Stoichiometry of the GABA\textsubscript{A} \(\alpha_1\beta_2\gamma_2\) Receptor

10103

employed for defining nonspecific binding in a radioligand binding assay.

Saturation Binding of EuK-c-Myc Antibodies to \(\alpha(c\text{-Myc})\beta_2\gamma_2\), \(\alpha_1\beta_2(c\text{-Myc})\gamma_2\), and \(\alpha_1\beta_2\gamma_2(c\text{-Myc})\)—Saturation binding experiments were carried out using control cells expressing \(\alpha_2\beta_2\gamma_2\) and cells expressing receptors where either the \(\alpha\), \(\beta\), or \(\gamma\)-subunit was epitope-tagged with c-Myc. Nonspecific binding of EuK c-Myc to control cells expressing an untagged receptor was subtracted from total binding at each concentration of antibody and was routinely 50% at 3 nM antibody. In all cases binding was saturable and had a high affinity, and EuK-labeled c-Myc bound to more sites in the \(\alpha_1\text{(c-Myc)}\beta_2\gamma_2\) and \(\alpha_1\beta_2\text{(c-Myc)}\gamma_2\) compared with \(\alpha_2\beta_2\gamma_2\text{(c-Myc)}\) as exemplified in Fig. 3. To quantitate this, the experiment was repeated on six separate occasions using 3 nM antibody, and the specific fluorescence counts are shown in Fig. 4A. The ratio of fluorescence labeling to control cells expressing an untagged receptor was both significantly higher than that observed with \(\alpha(c\text{-Myc})\beta_2\gamma_2\) and \(\alpha_1\beta_2\gamma_2(c\text{-Myc})\) was compared as shown in Fig. 4B. This clearly shows that there are twice as many EuK c-Myc sites on receptors where the \(\alpha\) or \(\beta\)-subunit is tagged compared with receptors where the \(\gamma\)-subunit is tagged (ratio of \(\alpha_1\text{(c-Myc)}\beta_2\gamma_2:\alpha_2\beta_2\gamma_2(c\text{-Myc})\gamma_2: \alpha_1\beta_2\gamma_2(c\text{-Myc}) = 1.89 \pm 0.12:1.94 \pm 0.18:1."

A comparison of the \(B_{\text{max}}\) values for \(^{3}\text{H}\text{Ro15-1788}\) and EuK-c-Myc fluorescence can be used to calculate the number of binding sites for the antibody/benzodiazepine binding site. For \(\alpha(c\text{-Myc})\beta_2\gamma_2\), there are 315 \pm 37 fluorescence counts/fmol of Ro15-1788 binding sites using 3 nM antibody and 1.8 nM \(^{3}\text{H}\text{Ro15-1788}\) (Fig. 4). Using Clarke’s equation (occupancy = [ligand]/[ligand] + \(K_d\)) this is equivalent to 610 \pm 71 fluorescence counts/fmol of Ro15-1788 binding sites. Given that there are 418 fluorescence counts of EuK/fmol of antibody (see “Experimental Procedures”), there are calculated to be 1.46 \pm 0.16 antibody binding sites/\(^{3}\text{H}\text{Ro15-1788}\) binding site. The limitations and multiple sources of error in this experiment (e.g. aggregation of antibody, heterogeneity of antibody labeling, etc.) would be more likely to lead to an underestimate of the number of antibody binding sites therefore the observation that there is more than one antibody binding site/\(^{3}\text{H}\text{Ro15-1788}\) binding site supports the most likely stoichiometry of 2 \(\alpha\)-subunits/receptor:1 benzodiazepine site.

Fluorescence Energy Transfer Studies—The affinity of the EuK-derivatized c-Myc antibodies for the GABA\textsubscript{A} subunits was 4–6 nM as shown in Fig. 3. A concentration of 3 nM was selected for the fluorescence transfer studies because this would less than half-maximally saturate the receptor. Following incubation with 3 nM EuK-derivatized c-Myc mAb, cells were washed and incubated with excess XL665-derivatized c-Myc mAb, and fluorescence at 665 nm was measured in response to laser excitation. As shown in Fig. 4C, a specific signal is observed, indicating energy transfer when either the \(\alpha\)- or the \(\beta\)-subunit is epitope-tagged (16.7 \pm 1.5 counts/fmol and 17.8 \pm 1.4 counts/fmol) but not when the \(\gamma\)-subunit is epitope-tagged. Because energy transfer can only take place when there are two c-Myc epitopes in close proximity, i.e. two subunits in the same receptor complex, this confirms that the stoichiometry of the GABA\textsubscript{A} \(\alpha_1\beta_2\gamma_2\) receptor under study is \((\alpha_1\beta_2)^2\gamma_2\). Further, the lack of any observable signal with \(\alpha_1\beta_2\gamma_2(c\text{-Myc})\) could be interpreted as evidence that receptors are not primarily expressed as clusters on the cell surface. If this were the case, it may be possible to observe energy transfer between \(\gamma\)-subunits on separate receptors.

**DISCUSSION**

To date, the stoichiometry of the GABA\textsubscript{A} receptor has been approached both directly and indirectly. Many laboratories have observed that a receptor can contain two different types of \(\alpha\)-subunits (16–20). Conflicting evidence has been obtained on accounted for the fluorescence in the absence of added EuK, and this was typically 800–1500 counts/s as shown in Fig. 2A. Binding of EuK c-Myc to cells expressing untagged receptors was significant (Fig. 2A). Efforts were made to try to reduce this nonreceptor-mediated binding by extensive washing following incubation with EuK-c-Myc mAb and preincubation of cells with IgG to block any IgG binding sites on the cells. Neither of these measures reduced nonspecific binding to control cells by more than 10% and were not routinely adopted. Instead, each experiment contained a wild type control (i.e. receptors expressed without any c-Myc epitope present), and nonspecific binding to these cells was subtracted from the total fluorescence signal, which was analogous to the methodology
whether two types of γ-subunit can coexist in a single receptor monomer with two studies proposing that two γ-subunits can be present (21, 22). There have been fewer studies on the presence of two β-subunits because the structural similarity of the β-subunits has precluded the development of antibodies, which clearly distinguish between them. 

There have been four studies that directly analyzed the stoichiometry of the receptor. The first two, chronologically, have taken an electrophysiological approach. Backus et al. (23) favor the composition 2α, 1β, and 2γ based on measuring the outward current induced by point mutation of charged amino acids on either side of the TM2 domain. Chang et al. (24) used a similar method (mutation of a lysine in TM2) to increase the sensitivity of the receptor to GABA in proportion to the number of mutant subunits present. In contrast to Backus et al. (23), they conclude that the stoichiometry is 2α, 2β, and 1γ, as do Im et al. (25) from studies of tandem subunit constructs. The most recent study used antibody labeling of chimeric subunits to determine the ratio of subunits present from Western blots, and they conclude that the structure is also 2α, 2β, and 1γ (11).

The approach taken here has used fluorescently derivatized mAbs to quantify subunits relative to the benzodiazepine binding site on the receptor and fluorescent resonance energy transfer to confirm that the stoichiometry of the receptor is (α1β2γ2). This method has several advantages over other previously described methods. 1) It uses intact cells; therefore only receptors that are expressed on the surface (and are therefore presumed to be correctly assembled) are considered (12, 26). 2) It involves minimal disruption of receptor structure by requiring only epitope tagging, which does not affect the expression or function of the receptor (12). 3) Only small amounts of transiently transfected material are required.

Furthermore this approach has general applicability to other multisubunit cell surface proteins and can be used particularly to investigate the stoichiometry of subunits in GABA<sub>A</sub> receptors composed of rarer subunits and in other ligand-gated ion channels.

Acknowledgments—We thank Drs. Eric Trinquet and Gerard Mathis of Cis-Bio International for constructive discussion, Dr. Mark Shearman for contributing to the assay development, and Peter Wingrove for providing the transfected cells.

REFERENCES
1. Mathis, G. (1995) Clin. Chem. 41, 1391–1397
2. Thomas, D. D., Carlsson, W. F., and Stryer, L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5748–5750
3. Damjanovich, S., Bene, L., Abdelkrim, A., Goldman, C. K., Sharrow, S., and Waldmann, T. A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 13134–13139
4. Raftery, M. A., Hunkapiller, M. W., Strader, C. D., and Hood, L. E. (1980) Science 208, 1454–1457
5. Cooper, C. E., Couturier, S., and Ballivet, M. (1991) Nature 350, 235–238
6. Mamalaki, C., Barnard, E. A., and Stephenson, F. A. (1989) J. Neurochem. 52, 124–134
7. Hadingham, K. L., Harkness, P. C., McKernan, R. M., Quirk, K., Le Bourdelles, B., Horne, A. L., Kemp, J. A., Barnard, E. A., Ragan, C. I., and Whiting, P. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 6378–6382
8. Nayeem, N., Green, T. P., Martin, I. L., and Barnard, E. A. (1994) J. Neurochem. 62, 815–818
9. Pritchett, D. B., Suntheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R., and seeburg, P. H. (1989) Nature 338, 582–585
10. McKernan, R. M., and Whiting, P. J. (1996) Trends Neurosci. 19, 139–143
11. Tretter, V., Ekya, N., Fuchs, K., and Sieghart, W. (1997) J. Neurosci. 17, 2728–2737
12. Connolly, C. N., Krishek, B. J., McDonald, B. J., Smart, T. G., and Mott, S. J. (1996) J. Biol. Chem. 271, 89–96
13. Wingrove, P. B., Wafford, K. A., Bain, C., and Whiting, P. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 4569–4573
14. Evan, G. I., Lewis, G., Ramsay, G., and Bishop, J. L. (1985) Nature 315, 525–531
15. Luddens, H., Killisch, L., and seeburg, P. H. (1991) J. Recept. Res. 11, 525–531
16. Zezula, J., and Sieghart, W. (1991) FEBS Lett. 284, 15–18
17. Mertens, S., Benke, D., and Mohler, H. (1993) J. Biol. Chem. 268, 5965–5973
18. McKernan, R. M., Quirk, K., Prince, R., Cox, P. A., Gillard, N. P., Ragan, C. I., and Whiting, P. (1991) Neuron 7, 667–676
19. Khan, Z. U., Gutierrez, A., and De Blas, A. L. (1994) J. Neurochem. 63, 1466–1476
20. Quirk, K., Gillard, N. P., Ragan, C. I., Whiting, P. J., and McKernan, R. M. (1994) Mol. Pharmacol. 45, 1061–1070
21. Backus, K. H., Arijoni, M., Drescher, U., Sehrer, L., Malherbe, P., Mohler, H., and sensen, J. A. (1993) Neuropeptide 5, 285–288
22. Chang, Y., Wang, R., Barot, S., and Weiss, D. S. (1996) J. Biol. Chem. 271, 5415–5424
23. Im, W. B., Prezenger, J. F., Binder, J. A., Dillen, G. H., and alberts, G. L. (1995) J. Biol. Chem. 270, 26063–26066
24. Angelotti, T. P., and macdonald, R. L. (1993) J. Neurosci. 13, 1429–1440