Repression of γ-Aminobutyric Acid Type A Receptor α1 Polypeptide Biosynthesis Requires Chronic Agonist Exposure*

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Jorge D. Miranda‡§ and Eugene M. Barnes, Jr.¶

From the ¶Neuroscience Division and §Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030

Although it is well established that the number of γ-aminobutyric acid type A (GABA_A) receptors declines in cortical neurons exposed to GABA_A receptor agonists, the mechanisms responsible for this use-dependent down-regulation remain unclear. Two hypotheses have been proposed: (i) agonist-evoked sequestration and degradation of surface GABA_A receptors and (ii) repression of receptor subunit biosynthesis. We have addressed this problem using [35S]Met/Cys pulse-chase labeling of chick cortical neurons in culture and immunoprecipitation and immunoblotting with an antibody (RP4) directed against a GABA_A receptor 35-kDa α1 polypeptide, but this rate declined by 35% after a 7-day treatment. This is consistent with a previous report (Baumgartner, B. J., Harvey, R. J., Darlison, M. G., and Barnes, E. M. (1994) Mol. Brain Res. 26, 9–17) that a 7-day GABA treatment of this preparation produced a 45% reduction in the α1 subunit mRNA level, while a 4-day exposure had no detectable effect. On the other hand, after a 4-day exposure to these agonists, a 30% reduction in the level of the α1 polypeptide was observed on immunoblots, similar to that found previously for down-regulation of GABA_A receptor ligand-binding sites. Thus, the de novo synthesis of GABA_A receptor α1 subunits is subject to a delayed use-dependent repression that was observed after, rather than before, the decline in neuronal levels of the polypeptide.

Pulse-chase experiments showed a monophasic degradation of the GABA_A receptor 35S-α1 subunit with a 1/2t = 7.7 h, a process that was unaffected by the addition of GABA to neurons during the chase period. These nascent 35S-labeled polypeptides are presumably diluted into the neuronal pool of unlabeled unassembled α1 subunits, which was found to exceed by a 4:1 molar ratio the amount assembled into [3H]flunitrazepam binding sites. Thus, the data reveal an alternative scheme for degradation of GABA_A receptor polypeptides: a pathway that may participate in the agonist-independent degradation of unassembled receptor subunits. This differs from another pathway for the agonist-dependent degradation of mature GABA_A receptors derived from the neuronal surface (Calkin, P. A., and Barnes, E. M., Jr. (1994) J. Biol. Chem. 269, 1548–1553).

γ-Aminobutyric acid type A (GABA_A) receptors are the major transducers of fast synaptic inhibition in the central nervous system and represent sites of action for anxiolytic and hypnotic drugs including benzodiazepines and barbiturates (1). There are four classes of GABA_A subunits (α, β, γ, and δ), and three of these have multiple members (α1–α6, β1–β4, γ1–γ4) (2, 3). Although the exact composition and stoichiometry of GABA_A Rs is still uncertain, it is believed that the major form of the native protein is composed of α, β, and γ subunits (4, 5) arranged in a pentamer that encloses a central chloride channel (6).

Prolonged occupancy of GABA_A Rs by agonists evokes a series of regulatory mechanisms that control the function, subcellular distribution, and number of receptors. Such use-dependent mechanisms can be partially distinguished by their temporal order, a paradigm used to describe down-regulation of G-protein-linked receptors (7). For GABA_A Rs, the most rapid of these events is desensitization, which is characterized by a decline in the frequency of channel openings occurring within a few s after GABA application (8). The kinetics of desensitization in isolated membrane patches show properties that are probably intrinsic to GABA_A Rs rather than a result of extrinsic modification. Within 1–2 h of exposure of neurons to GABA or benzodiazepines, [3H]flunitrazepam binding sites (9) and GABA_A Rs 125I-polypeptides (10) are sequestered from the cell surface. β-carboline-evoked internalization of GABA_A Rs binding has also been described (11). The pathway for use-dependent GABA_A receptor sequestration is not yet defined, but clathrin-coated vesicles have been implicated as a vehicle (12, 13). Following agonist-dependent internalization, GABA_A Rs polypeptides appear to be rapidly degraded (10). Much of the interest in these and related processes is due to a possible role in the development of tolerance and habituation to benzodiazepines and GABA mimetic drugs, a major problem in clinical applications (5, 13).

It is well established that chronic (several days) exposure of primary neuronal cultures to GABA reduces the density of high affinity binding sites for GABA_A Rs (14–18) and the magnitude of GABA-gated Cl⁻ currents (14, 16), a process referred to as down-regulation. In this article we continue to use the term “down-regulation” to refer specifically to the agonist-evoked decline in the number of assembled plasma membrane receptors. Down-regulation is accompanied by reductions in GABA_A Rs 125I-polypeptides (10) and in α-subunit immunoreactivity (19, 20). Two distinct mechanisms have been proposed for this use-dependent GABA_A down-regulation: (i) repression of GABA_A subunit biosynthesis (21) and (ii) degradation of GABA_A Rs subunits initiated by endocytosis (10). While there is general agreement that expression of GABA_A Rs subunit mRNAs declines as a result of GABA exposure (19,
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21–23), the relationship of this repression to receptor down-regulation is unclear. Although Montpied et al. (21) reported that GABA<sub>AR α1</sub>- and α2-subunit transcripts were reduced coordinately with [3H]flunitrazepam binding, Baumgartner et al. (22) found that the decline in [3H]flunitrazepam binding preceded that for the α1 subunit mRNA. To examine these issues further, we have employed [<sup>35</sup>S]Met/Cys labeling to measure the synthesis and degradation of nascent GABA<sub>AR α1</sub> subunit polypeptides in chick cortical neurons. The results are in accord with previous studies of the GABA<sub>AR</sub> receptor α1 subunit transcript in these cells (22), showing that repression of α1 subunit biosynthesis became detectable long after the GABA-evoked loss of the polypeptide.

**EXPERIMENTAL PROCEDURES**

Preparation and Characterization of Polyclonal Antibody RP4—A fusion protein containing a region of the chick GABA<sub>AR</sub> receptor α1 subunit, corresponding to amino acid residues 331–381 (24), was cloned in pRSET-B, expressed in *Escherichia coli* JM109, and purified by Ni<sup>2+</sup>-affinity chromatography according to the general procedures of Kroll et al. (25). This protein was used for rabbit immunizations, and the resulting RP4 antiserum was characterized by immunoprecipitation and Western blot analysis. Details of these procedures and the antibody characterization appear elsewhere (26, 27). A crude IgG fraction, prepared by elution of the antisera from CM Affi-Gel Blue (Bio-Rad), was absorbed on Affi-Gel 10 (Bio-Rad) derivatized with the α1-(331–381) fusion protein. The antibody was eluted with 4 M MgCl<sub>2</sub> (pH 6.4), dialyzed against TBS (20 mM Tris—Cl, 150 mM NaCl, pH 7.4), and concentrated.

Immunoprecipitation and Immunoblotting—The procedures of Tehrani and Barnes (28) were used for extraction and immunoprecipitation of central [3H]flunitrazepam binding sites from chick cortical neurons. Western blotting was performed according to Calkin and Barnes (10) using 1 μg/ml purified RP4 antibody or a 1:100 dilution of RP4 antisera.

Cell Culture and <sup>35</sup>S Labeling—Primary cultures of embryonic chick cortical neurons were prepared according to Thamby et al. (29). Where indicated, GABA or other test compounds were added 3 h after plating the cells. Neurons cultured for 4–7 days in 60-mm Petri dishes were washed and incubated for 15 min at 37 °C in Dulbecco’s modified Eagle’s medium lacking Met and Cys. The cells were pulsed by the addition of fresh medium containing 200 μCi/ml [<sup>35</sup>S]Met/Cys (Translabel, NEN Life Science Products) and GABA<sub>AR</sub> receptor ligands (where indicated) and returned to the incubator for 2 h. For chase experiments, the cells were washed three times with Dulbecco’s modified Eagle’s medium containing 2 mg/ml each of Met and Cys, and then incubated in conditioned medium containing 1 mM Met, 1 mM Cys, and 200 μM GABA (where indicated) for 3–48 h. Incubations were terminated by washing the cells with ice-cold PBS (137 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl<sub>2</sub>, (where indicated) for 3–48 h. Incubations were terminated by washing the cells with ice-cold PBS (137 mM NaCl, 2.68 mM KCl, 0.49 mM MgCl<sub>2</sub>, 0.9 mM CaCl<sub>2</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) containing 1 mM Met and 1 mM Cys. The cells were collected and homogenized in extraction buffer (2 mM EDTA, 2 mM EGTA, 1 mM Met, 1 mM Cys, 2 mM benzamidine, 0.1 mg/ml bacitracin, 0.1 mg/ml each of trypsin inhibitors II-O and II-S, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml phosphoramidon in TBS). The homogenates were centrifuged for 1 h at 100,000 × g, and the pellets were resuspended in extraction buffer containing 1% Triton X-100 and 0.1% SDS. After 30 min on ice, the extracts were clarified by centrifugation at 100,000 × g for 1 h and precleared twice with *Staphylococcus aureus* cells, and aliquots were withdrawn for determination of total <sup>35</sup>S incorporation by precipitation with 10% trichloroacetic acid. The trichloroacetic acid precipitates were washed with 70% ethanol and then washed with ether and counted. The remainder of the extract was used for double immunoprecipitation as described by Firestone and Winguth (30). Both immunoprecipitations utilized overnight incubations with 1 μg of affinity-purified RP4 antibody. The precipitates were analyzed on 10% polyacrylamide-SDS gels. The gels were prepared for autoradiography as described by Skinner and Grissowd (31).

Quantitative Densitometry—Autoradiographic densities from the pulse-chase and immunoblotting experiments were determined by using a laser scanner and Scan Analysis software (Biosoft, Ferguson, MO) following the procedures described by Kendrick et al. (32). The linear range of analysis for both types of experiments was determined by application of a series of sample dilutions to the gel. Linear regression analysis of the resulting calibration curves gave correlation coefficients >0.999, and all densities were quantified within this linear range of analysis. Student’s two-tailed t test was used for statistical evaluation of the data.

**RESULTS**

Immunoblotting and Immunoprecipitation of GABA<sub>AR</sub> Receptor α1 Subunits—For identification and quantitation of GABA<sub>AR</sub> receptor α1 subunits from chick cortical neurons in culture, we utilized an affinity-purified polyclonal antibody (RP4) directed against an α1-(331–381) fusion protein. On Western blots (Fig. 1), this antibody reacted with a single 51-kDa neuronal polypeptide, the mass expected for the GABA<sub>AR</sub> α1 subunit (33, 34). After preabsorption with the α1-(331–381) fusion protein, antibody RP4 did not give detectable labeling of the blots. Cross-reactivity with proteins in the 53–66-kDa range expected for GABA<sub>AR</sub> α2–α6 subunits (35) was not observed. Treatment of the neuronal membranes with endoglycosidase H caused a shift in the apparent mass of the immunoreactive polypeptide from 51 to 48 kDa (not shown), the latter corresponding to the value found for the unglycosylated GABA<sub>AR</sub> α1 subunit (35). Additional experiments (not shown) established that 58 ± 3% of the [3H]flunitrazepam binding sites extracted from 100 μg of neuronal membrane protein were precipitated at a saturating level (1 μg) of RP4 antibody. Similar values were obtained with membrane extracts from chick cerebral cortex.

To estimate the molar quantities of the GABA<sub>AR α1</sub> subunit produced by the neurons under these conditions, we utilized a affinity-purified polyclonal antibody (RP4) directed against an α1-(331–381) fusion protein as a standard for quantitative Western blotting (Fig. 2). These calibration curves were then used for the analysis of densities for the native α1 subunit obtained from immunoblots similar to those in Fig. 1. When applied to gels along with the standards, membrane proteins (10–50 μg) from 4-day cultures gave signals for the native 51-kDa α1 subunit (not shown) that were within the linear range of densitometric analysis as illustrated in Fig. 2. By this approach we obtained for the neuronal α1 subunit a
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![Graph](image)

**Fig. 2.** Quantitative immunoblotting of GABA<sub>A</sub>R α1-(331–381) fusion protein. The amounts shown of the α1-(331–381) fusion protein were analyzed by Western blotting as in Fig. 1 except that 15% polyacrylamide-SDS gels were used. From a series of such immunoblots, the autoradiographic intensity of the 9-kDa band was quantified by laser-scanning densitometry. The results are expressed as the mean ± S.E. from three experiments. The correlation coefficient for the least squares line shown is 0.999. From the three individual experiments, the mean ± S.E. of the correlation coefficients is 0.9943 ± 0.0032.

Use-dependent Decline in GABA<sub>A</sub> Receptor α1 Subunit Immunoreactivity—Quantitative Western blotting was also used to examine the modulation of GABA<sub>A</sub>R α1 subunit polypeptides during the exposure of neuronal cultures to receptor agonists. GABA (200 μM) was added 3 h after plating neurons, and the cells were cultured for 4 days. With the chick cortical preparation, similar procedures were employed previously to examine the use-dependent decline of GABA<sub>A</sub>R ligand binding sites, [125I]-polypeptides, and subunit mRNAs (10, 14, 22). The concentration of GABA (200 μM) used in these experiments was also shown to be nearly saturating for down-regulation (14).

Under these conditions, chronic exposure to GABA or to isoguvacine, a GABA<sub>A</sub>-specific agonist, produced a decline in the level of the 51-kDa immunoreactive polypeptide (Fig. 3). Densitometric analysis of a series of such experiments revealed an attenuation of 27–32% in the presence of either ligand. These effects of GABA and isoguvacine were statistically significant (p < 0.02). Since isoguvacine is not a substrate for GABA transport or GABA transaminase, the data indicate that neuronal uptake or degradation does not limit the effectiveness of GABA in these experiments. The decline in α1 subunits produced by GABA was blocked by the addition of the GABA<sub>A</sub>-specific antagonist R5135 (3α-hydroxy-16-imino-5β-17-aza-androstan-11-one), indicating the necessity of receptor occupancy. R5135 was shown previously to block the GABA<sub>A</sub>-evoked sequestration and down-regulation of GABA<sub>A</sub> receptors (10).

Repression of GABA<sub>A</sub> Receptor α1 Subunit Biosynthesis—To measure the relative rates of GABA<sub>A</sub>R α1 subunit biosynthesis, the neurons were pulsed for 2 h in culture with 35S-Met/Cys, and extracts were prepared from 100,000 x g pellets of crude membranes. The extracts were analyzed by double immunoprecipitation with affinity-purified RP4 antibody and separation of the precipitates on SDS gels. As shown in Fig. 4, a major

![Graph](image)

**Fig. 3.** Quantitative immunoblotting of membranes from neurons chronically exposed to GABA<sub>A</sub>R ligands. Upper panel, neurons were cultured for 4 days without additions to the medium (C) or with 200 μM GABA (G), 200 μM GABA plus 1 μM R5135 (G + R), 1 μM R5135 (R), or 200 μM isoguvacine (I). Neuronal membrane proteins (20 μg) were analyzed by Western blotting as in Fig. 1 except that RP4 antiserum was used at a 1:100 dilution. Lower panel, from a series of Western blots prepared as in the upper panel, the autoradiographic intensity of the 51-kDa band was quantified as in Fig. 2. The linear range for quantitation was established by application of a series of membrane protein concentrations (not shown). The results are expressed as a percentage of the density of the 51-kDa band from untreated cells and represent the mean ± S.E. from four culture preparations. *, p < 0.02 by t test relative to the GABA plus R5135 control.
noteworthy that, as illustrated in Fig. 6, a 4-day exposure of the cells to GABA or isoguvacine failed to significantly repress de novo synthesis of the α1 polypeptide. Although the S.E. values in Fig. 6 were similar to those in Fig. 4, p values of 0.574 and 0.548 were obtained for the level of 35S-α1 polypeptides found in cells cultured for 4 days with GABA or isoguvacine, respectively, relative to the control (Fig. 6). Likewise, when neurons were cultured for 4 or 7 days in the absence of agonist, the presence of GABA during a 2-h pulse did not alter the level of 35S incorporation into this subunit (not shown). This indicates the lack of an acute effect of GABA that could have faded during the chronic exposures.

Turnover of GABA<sub>A</sub> Receptor α1 Subunits—To determine the rate of GABA<sub>A</sub> R α1 subunit degradation, neurons from 4-day cultures were pulsed for 2 h with [35S]Met/Cys and then chased in conditioned media for 3–48 h. The level of the 35S-α1 polypeptide declined continuously during the first 24-h period and was barely detectable thereafter (Fig. 7). Densitometric analysis of three such experiments yielded a t<sub>1/2</sub> = 7.7 ± 0.8 h for the α1 subunit. The general pool of neuronal membrane 35S-proteins was much more stable, declining with an apparent t<sub>1/2</sub> > 24 h. Since previous studies suggested a GABA-evoked degradation of GABA<sub>A</sub> receptor 32P-polypeptides derived from the cell surface (10), the effect of adding GABA to the chase medium was examined. However, the presence of GABA during the chase period had no discernible effect on the rate of degradation of either the GABA<sub>A</sub> R 35S-α1 subunit or the membrane protein pool.

DISCUSSION

Use-dependent Decline of GABA<sub>A</sub> Receptor α1 Subunit Immunoreactivity—The α1 subunit is a major component of GABA<sub>A</sub>Rs in most brain regions and an important determinant for receptor pharmacology. It also forms part of the high affinity site for benzodiazepine binding (36, 37). Although there have been multiple reports of a decline in GABA<sub>A</sub> R α1 subunit mRNA levels that are evoked by exposure of neurons to GABA agonists (21–23), there is little related information about the corresponding polypeptide. Although Calkin and Barnes (10)
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found that chronic exposure of chick cortical neurons to GABA agonists produced a decline in GABA\textsubscript{\textalpha}R \textalpha1 polypeptides, the \textalpha1 subunit was only tentatively identified as part of this labile pool. In the present paper, we show that a 27–32% decline of GABA\textsubscript{\textalpha}R \textalpha1 subunit immunoreactivity was produced by 4-day agonist treatments. This reduction is somewhat less than that found for \textsuperscript{[3]H}flunitrazepam binding (31–50%) under comparable conditions (10, 14). However, at saturation with the \textalpha1 subunit antibody, 58 ± 3% of the \textsuperscript{[3]H}flunitrazepam binding sites were precipitated, suggesting that only this fraction of the binding sites contains \textalpha1 subunits. The GABA-evoked decline in \textalpha1 subunits was prevented by R5135, a GABA antagonist shown to be effective against various subtypes of native GABA\textsubscript{\textalpha}Rs (38). Alone, R5135 had little effect, showing that endogenous GABA makes little contribution in these experiments. Isovagucine, a GABA\textsubscript{\textalpha}R-specific agonist that is not subject to degradation or uptake by neurons, produced a decrease of \textalpha1 subunit immunoreactivity that was equivalent to that produced by GABA. Thus, the first conclusion to be drawn is that nascent GABA\textsubscript{\textalpha}R receptor \textalpha1 subunits are degraded by an agonist-independent pathway. As discussed below, we believe that this differs from another, previously described pathway for the agonist-dependent degradation of mature GABA\textsubscript{\textalpha}R receptors derived from the cell surface (10).

Although the turnover of GABA\textsubscript{\textalpha}R \textalpha1 subunits in our pulse-chase technique in the chick cortical neuron cultures we found a monophasic decline in the \textsuperscript{35}S-\textalpha1 polypeptide with a \textit{t\textsubscript{1/2}} = 7.7 h. Exposure of the neurons to GABA during the 24-h chase period had no discernible effect on the degradation of the \textsuperscript{35}S-\textalpha1 subunit. Thus, the third conclusion from our experiments is that nascent GABA\textsubscript{\textalpha}A receptor \textalpha1 polypeptides are degraded by an agonist-independent pathway. As discussed below, we believe that this differs from another, previously described pathway for the agonist-dependent degradation of mature GABA\textsubscript{\textalpha}R receptors derived from the cell surface (10).

FIG. 7. Pulse-chase analysis of GABA\textsubscript{\textalpha}R \textalpha1 polypeptide degradation. Upper panel, neurons were cultured for 4 days without test compounds and \textsuperscript{35}S-labeled as in Fig. 4. The cells were chased in conditioned medium in the absence (C) or presence of 200 \mu M GABA (G) for the times shown. Membrane extracts containing an equivalent amount of protein were immunoprecipitated and analyzed as in Fig. 4. Only the \textsuperscript{35}S-\textalpha1 subunit (51-kDa) band is shown. Lower panel, autoradiographic densities from a series of such experiments were determined as in Fig. 2. Data are shown for the \textalpha1 subunit from cells chased in the absence (○) or presence of GABA (●). Solvent-washed trichloroacetic acid precipitates from \textsuperscript{35}S-membrane extracts were counted, and the data were normalized per milligram of protein and shown for cells chased in the absence (○) or presence of GABA (●). The results are expressed as a percentage of values for unchased cells and represent the mean ± S.E. from three culture preparations.

Repression of GABA\textsubscript{\textalpha}A Receptor \textalpha1 Subunit Biosynthesis—To examine the mechanism of the use-dependent decline in \textalpha1 subunits, we measured rates of \textsuperscript{35}S-Met/Cys incorporation by immunoprecipitation. Since these experiments were conducted with \textsuperscript{35}S pulses whose duration was within the linear range of incorporation (Fig. 5), the results may be taken to reflect the initial rates of \textalpha1 subunit synthesis \textit{de novo}. To our knowledge, these are the first such measurements for any GABA\textsubscript{\textalpha}R subunit. Neither acute treatment of the cells with GABA during the 2-h \textsuperscript{35}S pulse nor prior chronic treatment for 4 days had a detectable effect on \textalpha1 subunit synthesis. However, after 7 days of exposure to GABA or isoguvacine, a significant decline (33%) in the rate of \textalpha1 subunit translation was observed. This use-dependent repression was also prevented by R5135. These findings are in good agreement with previous measurements of GABA\textsubscript{\textalpha}R \textalpha1 subunit mRNA levels under comparable conditions in this preparation (22). GABA administration to the neurons for 7 days produced a significant reduction (47 ± 8%) in the level of \textalpha1 subunit mRNA, while a 4-day treatment had no effect. That the \textalpha1 subunit mRNA represents a sensitive indicator for repression is indicated by an equivalent agonist-dependent decline in GABA\textsubscript{\textalpha}R \textalpha1, \beta2, \beta4, and \textgamma2 subunit mRNAs (22). Therefore, our current findings support a delayed use-dependent repression of GABA\textsubscript{\textalpha}A receptor subunit biosynthesis. It is apparent that this repression is evoked only after a long delay, long after the neuronal pool of GABA\textsubscript{\textalpha}A receptor \textalpha1 subunits and assembled receptors has declined. This notion is also consistent with studies of GABA\textsubscript{\textalpha}R agonist administration \textit{in vivo}, which show a loss of receptor binding sites before detectable changes in pools of GABA\textsubscript{\textalpha}R subunit mRNAs (39, 40). Although it is conceivable that small reductions in GABA\textsubscript{\textalpha}R \textalpha1 subunit biosynthesis occurring within the first 4 days of GABA exposure could have escaped detection (cf. Fig. 6), the decline in the synthetic rate was readily measured after longer treatments (Fig. 4). Collectively, this leads to a second conclusion, that the biosynthesis of GABA\textsubscript{\textalpha}A receptor \textalpha1 subunits is subject to use-dependent repression, but this process was detectable only after, rather than before, receptor down-regulation.

Degradation of Nascent GABA\textsubscript{\textalpha}A Receptor \textalpha1 Subunits—To examine the turnover of GABA\textsubscript{\textalpha}R \textalpha1 subunits, we utilized a \textsuperscript{35}S pulse-chase technique. In the chick cortical neuron cultures we found a monophasic decline in the \textsuperscript{35}S-\textalpha1 polypeptide with a \textit{t\textsubscript{1/2}} = 7.7 h. Exposure of the neurons to GABA during the 24-h chase period had no discernible effect on the degradation of the \textsuperscript{35}S-\textalpha1 subunit. Thus, the third conclusion from our experiments is that nascent GABA\textsubscript{\textalpha}A receptor \textalpha1 polypeptides are degraded by an agonist-independent pathway. As discussed below, we believe that this differs from another, previously described pathway for the agonist-dependent degradation of mature GABA\textsubscript{\textalpha}A receptors derived from the cell surface (10).

Although GABA\textsubscript{\textalpha}A receptor \textsuperscript{35}S-subunit turnover has not been previously measured, the degradation of receptor polypeptides photolabeled with \textsuperscript{[3]H}flunitrazepam has been examined (41). Degradation of the photolabeled 48- and 51-kDa proteins in chick brain neurons was biphasic, showing \textit{t\textsubscript{1/2}} values of 3.8 and 32 h. Why do these values differ from the one obtained from our \textsuperscript{35}S measurements? We presume that the \textit{t\textsubscript{1/2}} values obtained by Borden et al. (41) reflect properties of assembled receptors because coexpression of GABA\textsubscript{\textalpha}R \textalpha1 and \textgammay subunits is necessary for \textsuperscript{[3]H}flunitrazepam binding (4). Furthermore, GABA\textsubscript{\textalpha}Rs assembled from \textalpha1, \beta2, and \textgammay subunits are transported to the cell surface, while unassembled \textalpha1 subunits are retained in the endoplasmic reticulum (35). It therefore appears likely that the pathways for degradation of unassembled and assembled \textalpha1 subunits differ as illustrated in Fig. 8. If the \textsuperscript{35}S-\textalpha1 polypeptides from our labeling studies are mostly unassembled, then differences in degradation pathways could account for the discordance of our data with that of Borden et al. (41).

Does the turnover of GABA\textsubscript{\textalpha}R \textalpha1 subunits in our pulse-chase measurements reflect unassembled or assembled subunits? Since our double immunoprecipitation procedure precludes a direct answer to this question, our argument that most of the nascent \textsuperscript{35}S-\textalpha1 subunits are unassembled relies on indirect evidence. From the quantitative immunoblotting experiments,
we estimate that cortical neurons, 4 days in culture, contain approximately 0.65 ± 0.11 pmol of α1 subunit/mg of membrane protein. Previous determinations of [3H]flunitrazepam binding density in these preparations gave a value of 0.22 ± 0.01 pmol/mg (14, 42). Since 58% of these [3H]flunitrazepam binding sites contain α1 subunits (i.e. immunoprecipitable by RP4 antibody in the present study), the density of receptors containing coassemblies of α1 and γ subunits is estimated to be 0.13 pmol/mg. By this approach, the ratio of unassembled to assembled GABA A receptor α1 subunits is approximately 4:1. This is consistent with quantitative reverse transcriptase-polymerase chain reaction data showing that the level of GABA A receptor α1 subunit mRNA greatly exceeds levels of the other subunits in these preparations (22). Thus, our data support the hypothesis that most of the α1 subunits in the cortical neuron cultures are unassembled, although further work is necessary to establish this conclusively. We believe that our chase experiments reflect the degradation of unassembled 35S-α1 polypeptides, which would presumably be diluted into a large pool of unlabelled α1 subunits retained in the endoplasmic reticulum.

Sequestration and Degradation of Surface GABA A Receptors—We have previously shown that an acute (4-h) exposure of cortical neurons to 100 μM GABA in culture produces an increase in the intracellular pool of [3H]flunitrazepam binding sites (9). This phenomenon could have been due to an increase in the synthesis of new receptors or a sequestration of existing receptors. Since the presence of GABA during the 35S pulse failed to influence GABA A receptor α1 subunit biosynthesis, our current findings lend support to a mechanism involving agonist-dependent sequestration of binding sites from the surface (9). Further studies by Calkin and Barnes (10) demonstrated that surface GABA A receptor 125I-polypeptides, including a probable α1 subunit, were sequestered during 2–4 h of GABA exposure. In the absence of GABA, sequestration was not observed. Although small, the sequestered pool of 125I-subunits was quite labile, having an approximate t1/2 of 2 h. However, during longer agonist exposures, the number of intracellular receptors stabilized, presumably due to replenishment from the surface, and the surface pool declined. If this decline in surface subunits cannot be accounted for by reductions in de novo synthesis, as our current findings indicate, they must have been degraded (cf. Fig. 8). Thus, the biosynthetic data described here help support the previous hypothesis that GABA A receptors are regulated by use-dependent sequestration and degradation.

Agonist-dependent degradation of surface GABA A receptors occurs, how could our 35S pulse/chase experiments fail to detect it? Our answer to this paradox is that assembly and transport of the nascent 35S-α1 subunits appears to be slow and inefficient. As discussed above, our data suggest that most of the α1 subunits in the neurons remain unassembled. Furthermore, of the newly synthesized/assembled sites photolabeled by [3H]flunitrazepam (Fig. 8, steps 1 and 2), only 4% reach the neuronal surface in 4 h (43). On this basis, we believe it is unlikely that a substantial fraction of nascent 35S-α1 subunits would have acquired a surface localization during the 24-h "window" of our chase experiments.

GABA A Receptor Down-regulation—Two hypotheses have been proposed to account for the use-dependent decline in surface GABA A receptors: (i) agonist-dependent sequestration and degradation of surface receptors (10) and (ii) repression of GABA A receptor subunit biosynthesis (21). While the findings in the current report are consistent with the operation of both mechanisms, our data show that hypothesis II cannot account for the onset of down-regulation. Repression of GABA A receptor α1 subunit biosynthesis was observed only after the detection of down-regulation. Following the initial binding of externally applied agonists, a small pool of surface GABA A receptors appears to be rapidly sequestered and degraded, while repression of the synthesis of new receptor subunits is a much slower process. The nature of the intracellular signals that link gene expression to the surface binding of agonists is not understood. The temporal order of these events prompts speculation that such signals could be provided by molecules made available by the sequestration/degradation pathway.

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