Analysis of the Presence and Abundance of GABA<sub>A</sub> Receptors Containing Two Different Types of α Subunits in Murine Brain Using Point-mutated α Subunits*  

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γ-Aminobutyric acid, type A (GABA<sub>A</sub>) receptors are pentameric proteins of which the majority is composed of two α subunits, two β subunits and one γ subunit. It is well documented that two different types of α subunits can exist in a single GABA<sub>A</sub> receptor complex. However, information on the abundance of such GABA<sub>A</sub> receptors is rather limited. Here we tested whether mice containing the His to Arg point mutation in the α1, α2, or α3 subunit at positions 101, 102, and 126, respectively, which render the respective subunits insensitive to diazepam, would be suitable to analyze this issue. Immunodepletion studies indicated that the His to Arg point mutation solely rendered the respective GABA<sub>A</sub> receptors totally insensitive to diazepam binding that contain two mutated α subunits in the receptor complex, whereas receptors containing one mutated and one heterologous α subunit did not carry the mutation remained sensitive to diazepam binding. This feature permitted a quantitative analysis of native GABA<sub>A</sub> receptors containing heterologous α subunits by comparing the diazepam-sensitive binding sites in mutant mouse lines containing one mutated α subunit with those present in mouse lines containing two different mutated α subunits. The data indicate that the α<sub>1</sub>α<sub>2</sub> containing receptors with 61% is the most abundant receptor subtype in brain, whereas the α<sub>1</sub>α<sub>3</sub> (13%), α<sub>1</sub>β<sub>2</sub> (15%), α<sub>2</sub>α<sub>2</sub> (12%), α<sub>2</sub>β<sub>2</sub> (8%), α<sub>2</sub>β<sub>3</sub> (9%), and α<sub>3</sub>β<sub>3</sub> combinations (4%) are considerably less expressed. Only within the α containing receptor population does the combination of equal α subunits (84% α<sub>1</sub>α<sub>1</sub>, 7% α<sub>1</sub>α<sub>2</sub>, and 8% α<sub>1</sub>α<sub>3</sub>) prevail, whereas in the α<sub>2</sub> containing receptor population (46% α<sub>2</sub>α<sub>2</sub>, 36% α<sub>2</sub>α<sub>1</sub>, and 19% α<sub>2</sub>α<sub>3</sub>) and particularly in the α<sub>3</sub> containing receptor population (27% α<sub>3</sub>α<sub>3</sub>, 56% α<sub>3</sub>α<sub>1</sub>, and 19% α<sub>3</sub>α<sub>2</sub>) the receptors with two different types of α subunits predominate. This experimental approach provides the basis for a detailed analysis of the abundance of GABA<sub>A</sub> receptors containing heterologous α subunits on a brain regional level.

GABA<sub>A</sub> receptors mediate the majority of fast inhibitory neurotransmission in the mammalian brain by controlling an integral chloride ion channel. Enhancement of neuronal inhibition by positive allosteric modulation of GABA<sub>A</sub> receptors via the benzodiazepine-binding site underlies the pharmacotherapy of several neurological and psychiatric disorders such as generalized anxiety disorders, sleep disturbances, and seizure disorders. GABA<sub>A</sub> receptors are pentameric transmembrane proteins build of different classes of subunits (α1–6, β1–3, γ1–3, ρ1–3, δ, ε, and θ) (1). The vast majority of GABA<sub>A</sub> receptors are composed of α, β, and γ subunits, whereas the α subunit variant determines the ligand binding characteristics of the benzodiazepine site of the various receptor subtypes. GABA<sub>A</sub> receptors containing the α1, α2, α3, or α5 subunit in combination with any β subunit and any γ subunit are the most abundant subtypes in the brain and mediate the modulatory actions of clinically used benzodiazepine site agonists, such as diazepam, flunitrazepam, and clonazepam (2, 3). The binding of clinical relevant benzodiazepine site ligands to these so-called diazepam-sensitive GABA<sub>A</sub> receptor subtypes depends on a single amino acid residue, His at position 101 in α1 and α2, position 126 in α3, or position 105 in α5 (4, 5).

It is well documented that GABA<sub>A</sub> receptors exist in brain that contain two different types of subunits in a single receptor complex (6–15). However, it is currently poorly understood to which degree receptors containing two heterologous α subunits contribute to diazepam-sensitive GABA<sub>A</sub> receptors. So far, the abundance of α1α2- and α1α3-containing receptors in the cerebral cortex and α1γ5- and α2γ5-containing receptors in the hippocampus have been estimated by sequential steps of immunoprecipitation and quantification of precipitated receptors by radioligand binding (6–8). This methodological approach is a demanding task and because of the amount of tissue required is hardly applicable to all brain regions. In addition, immunopurification studies are limited by the fraction of receptors that can be solubilized from the membranes.

In the present paper an alternative experimental approach was used to overcome these experimental limitations. This method is based on the analysis by ligand binding studies of mutant mouse lines carrying a point mutation in the diazepam-sensitive α subunits. Because the diazepam sensitivity of GABA<sub>A</sub> receptors depends on the presence of a histidine residue in the drug-binding domain of the α subunits α1, α2, α3, and α5, they are rendered diazepam-insensitive when this histidine residue is replaced by arginine (4, 5). This feature had been previously used to analyze the contribution of GABA<sub>A</sub> receptor subtypes to the diverse actions of diazepam. By generating mouse lines that contain the His to Arg (H/R) point mutation in the α1, α2, or α3 subunit, it was shown that sedation, antegrade amnesia, and most of the anticonvulsant activities are mediated by α1-containing receptors (16),
whereas the anxiolytic like activity is mediated by α2-containing receptors (17).

Here we show that the different α3(H126R) point-mutated mouse lines provide an opportunity to estimate the abundance of receptor populations containing two different types of α subunits in a receptor complex. Immunodepletion studies indicated that in α3(H126R) mutant mouse lines only receptors containing two mutated α subunits fail to bind diazepam (e.g. α1(H101R) α2(H101R), whereas receptors containing one mutated α subunit and one nonmutated heterologous α subunit (e.g. α1(H101R) α3) remain sensitive to diazepam binding. This permitted a detailed analysis on the presence and abundance of receptors containing either two homologous or two heterologous α subunits by comparing the proportion of diazepam-sensitive and diazepam-insensitive binding sites in wild type mice with the mouse lines containing a single type of mutated α subunit (α1H101R, α2H101R, or α3H126R) and newly crossbred mouse lines containing two different types of mutated α subunits (α1H101R α2H101R, α1H101R α3H126R, or α2H101R α3H126R).

MATERIALS AND METHODS

**Animals**—Mouse lines containing the α1H101R, α2H101R, or α3H126R point mutation (at least five backcrosses to the 129/SvJ background) were generated previously (16, 17). The double homozygous mutant mouse lines α1H101R α2H101R, α1H101R α3H126R, and α2H101R α3H126R were generated by cross-breeding the single-mutant mouse lines. Mice were kept under normal 12-h day-night cycle conditions with food and water ad libitum.

**Antibodies**—Antibodies selectively recognizing the α1 (18), α2 (19), or α3 subunit (18) had been previously generated against short amino acid sequences present only in the subunit of interest (α1, amino acids 421–429; α2, amino acids 418–424; and α3, amino acids 1–15). The antibodies had been thoroughly analyzed for subunit specificity by Western blotting, immunoprecipitation, and immunohistochemistry (for details see Refs. 9 and 18–20).

**Membrane Preparation**—Brain tissue from 8–12-week-old mice was homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and centrifuged at 1000 × g for 10 min. Following centrifugation of the supernatant for 20 min at 12,000 × g, the crude membrane pellet was washed three times with buffer and stored at −30°C until used.

**Solubilization and Immunoprecipitation**—For solubilization, the membranes were thawed and washed once with 10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM EDTA, 200 μg/ml bacitracin, 0.1 mM phenylmethylsulfonyl fluoride, 2.3 μg/ml aprotinin, 1 μM benzamidine and resuspended in the same buffer to a protein concentration of 5–7 mg/ml followed by addition of sodium deoxycholate to a final concentration of 0.5%. After incubation for 30 min at 4°C, insoluble material was removed by centrifugation for 60 min at 100,000 × g. The supernatant was immediately used for immunoprecipitation experiments.

For immunoprecipitation, aliquots (0.2–0.5 ml) of the deoxycholate leading extract were incubated with subunit-selective antisera at concentrations leading to the saturation of the respective antisera. The specificity of immunoprecipitation was verified in competition experiments using the respective peptide antigen (50 μg/ml extract). No specific [3H]Ro 15-4513 binding was observed in the immunoprecipitate after co-incubation of the respective antisera with the corresponding peptide antigen (data not shown).

[3H]Ro 15-4513 Binding Experiments—For Scatchard analysis, crude mouse brain membranes were thawed and washed once with 50 μM Tris-HCl, pH 7.5, and aliquots containing 100 μg of protein were incubated with increasing concentrations (0.5–70 nm) of [3H]Ro 15-4513 (2.2 Ci/mmol) in a total volume of 0.2 ml for 90 min on ice. Diazepam-insensitive binding sites were detected in parallel by inclusion of 10 μM diazepam in the incubation. Nonspecific [3H]Ro 15-4513 binding was assessed at each radioligand concentration by the addition of 10 μM flumazenil to the reaction. Incubation was stopped by rapid vacuum filtration on Whatman GF/C filters using a semi-automatic cell harvester (Skatron Instruments) and subjected to liquid scintillation counting.

Diazepam displacement studies using immunoprecipitated GABA<sub>A</sub> receptors were performed by incubating aliquots of the washed immunoprecipitates with serial dilutions of diazepam at a fixed concentration of [3H]Ro 15-4513 (10 nM). After incubation for 90 min on ice, the reaction was stopped by addition of 4 ml of 50 mM Tris-HCl, pH 7.5, followed immediately by rapid vacuum filtration on Whatman GF/C filters. The filters were washed three times with 4 ml of buffer and subjected to liquid scintillation counting. Nonspecific radioligand binding was determined by including 10 μM of flumazenil in parallel incubations. The binding data were analyzed using the program KELL for Windows 6.0.5 (Biosoft, UK).

**RESULTS**

Diazepam Binding to GABA<sub>A</sub> Receptors in Point-mutated Mice Containing α1H101R, α2H101R, or α3H126R Subunits—Generation of mouse lines containing the H/R point mutation in the α1, α2, or α3 subunit resulted in an increase of diazepam-insensitive [3H]Ro 15-4513-binding sites with a brain regional distribution that corresponded to the expression pattern of the respective α subunit in wild type mice (16, 17). In α1H101R mice the increase in diazepam-insensitive binding sites, determined by Scatchard analysis (61 ± 3% (16), corresponded roughly to the abundance of α1-containing receptors in whole brain (60–80%), as previously analyzed by immunoprecipitation (11, 14, 18, 21, 22). However, the increase was considerably smaller than expected in the α2H101R and α3H126R mice. In α2H101R and α3H126R mice the number of diazepam-insensitive binding sites increased by 12 ± 0.1 and 4 ± 2% (17), respectively, whereas the abundance of α2-containing receptors was immunobiochemically estimated to be 20–28% (11, 19, 22) and that for α3-containing receptors to 18–24% (8, 14, 18, 22). This mismatch between the increase of diazepam-insensitive binding sites in the α2H101R and α3H126R mouse lines and the abundance of α2- and α3-containing receptor populations suggests that the receptors that contained the respective point-mutated subunit were not rendered fully insensitive to diazepam binding.

To analyze this assumption, the respective α1H101R, α2H101R, and α3H126R-containing receptor populations were isolated by immunoprecipitation from deoxycholate extracts of whole brain membranes using subunit-selective antisera. The proportion of diazepam-sensitive and diazepam-insensitive [3H]Ro 15-4513-binding sites in the immunoprecipitated receptor populations was then determined by diazepam displacement of [3H]Ro 15-4513 binding. Interestingly, all of the receptor populations containing the point mutation displayed a high affinity diazepam-binding component corresponding to that of wild type receptors (Fig. 1). Whereas in α1H101R-containing receptors only 16 ± 1% remained diazepam-sensitive, 54 ± 10% of α2H101R-containing receptors and 73 ± 4% of α3H126R-containing receptors displayed diazepam-sensitive binding sites (Fig. 1 and Table II). This result demonstrates that at least some subpopulations of GABA<sub>A</sub> receptors containing the H/R mutation had retained diazepam-sensitive binding sites.

Because GABA<sub>A</sub> receptors exist in brain that contain two different types of α subunits in the same receptor complex (6–15), the presence of high affinity diazepam binding in the mutated receptor populations is likely to be caused by the subpopulation of GABA<sub>A</sub> receptors containing a nonmutated heterologous α subunit (which is not α4 or α6) in the receptor complex that confers high affinity diazepam binding. To verify this hypothesis, brain extracts were immunodepleted in a sequential fashion to initially remove receptors containing the nonmutated α subunit (Fig. 2). For instance, from the brain extract of α2H101R mice α1-containing receptors were removed by quantitative immunoprecipitation followed by precipitation of the receptor population containing the α2 subunit and de-
crude membranes were prepared from brains of wild type mice or mice
present in the point-mutated receptor populations are due to
ments demonstrate that the diazepam-sensitive binding sites
14%, respectively (Fig. 2). Thus, the immunodepletion experi-
—
creasing concentrations of diazepam. For wild type receptors,
receptors increased diazepam-insensitive binding in
2-containing receptors, GABAA receptors are known to contain two
heterologous α subunits. Indeed, in the brain extract from
2(H101R) mice immu-
2- and
3-containing receptors yielded similar displacement curves. The data
represent the means ± S.D. of three independent experiments. Error
bars smaller than the symbols are not shown.

DISCUSSION

GABA_A receptors are known to contain two α subunits that can be homologous or heterologous. If they are distinct they
represent a different class of receptor subtype. In the present paper we studied the presence and abundance of GABA<sub>A</sub> receptors containing two different types of <i>a</i> subunits based on an analysis of mice containing the H/R point mutation in the <i>a</i>1, <i>a</i>2, or <i>a</i>3 subunit. This point mutation rendered the respective subunits insensitive to diazepam binding.

**Basis and Prerequisites**—There are two prerequisites for using mouse lines containing the <i>a</i>1<sup>H101R</sup> mutation for a quantitative analysis of GABA<sub>A</sub> receptors containing two different types of <i>a</i> subunits. First, the introduction of the point mutation should not affect the expression levels and the distribution of the mutated subunit as well as those of the nonmutated subunits. It was shown previously that expression and targeting of receptor subunits appear not to be affected by the point mutation. Mice containing the H/R mutation in the <i>a</i>1, <i>a</i>2, or <i>a</i>3 subunit expressed the mutated subunit at normal levels with unaltered expression patterns and did not induce up-or down-regulation of other GABA<sub>A</sub> receptor subunits to an appreciable extent (16, 17).

Second, in receptors containing one mutated and one nonmutated <i>a</i> subunit, the nonmutated <i>a</i> subunit should confer diazepam-sensitive binding to the receptor complex. The first
GABA<sub>A</sub> Receptors Containing Two Heterologous α Subunits

**Table II**

Proportion of diazepam-sensitive and diazepam-insensitive [3H]Ro 15-4513-binding sites in GABA<sub>A</sub> receptor populations immunoprecipitated with a subunit-selective antiserum from brain extracts of the various single- and double-mutant mouse lines

| Genotype            | Antibody used for IP | Diazepam-sensitive binding sites | Diazepam-insensitive binding sites |
|---------------------|----------------------|----------------------------------|------------------------------------|
|                     |                      | % total                          | % total                            | % increase*                        |
| α<sub>1</sub>H101R  | a1                   | 16 ± 1                           | 84 ± 1                             |                                   |
| α<sub>2</sub>H101R  | a2                   | 54 ± 10                          | 46 ± 10                            |                                   |
| α<sub>3</sub>H101R  | a3                   | 73 ± 4                           | 27 ± 4                             |                                   |
| α<sub>1</sub>H101R  | α<sub>2</sub>H101R   | a1                               | 9 ± 7                              | 7                                 |
| α<sub>1</sub>H101R  | α<sub>3</sub>H101R   | a1                               | 18 ± 7                            | 36                                |
| α<sub>2</sub>H101R  | α<sub>3</sub>H101R   | a2                               | 8 ± 10                            | 8                                 |
| α<sub>2</sub>H101R  | α<sub>3</sub>H101R   | a3                               | 17 ± 2                            | 56                                |
| α<sub>1</sub>3H126R | a2                   | 35 ± 8                           | 65 ± 8                            | 19                                |
| α<sub>2</sub>3H126R | a3                   | 54 ± 13                          | 46 ± 13                            | 19                                |

* The values are compared to the respective single mutants. % increase refers to the diazepam-insensitive binding sites present in the double-mutant receptor (e.g., α<sub>1</sub>H101R α<sub>2</sub>H101R) precipitated with a1 antiserum: 91% minus the diazepam-insensitive binding sites of the corresponding single-mutant receptors (α<sub>1</sub>H101R; 84%). In this example, the difference in diazepam-insensitive binding sites (7%) corresponds to the abundance of α<sub>1</sub>α<sub>2</sub> receptors in the α<sub>1</sub> receptor population. The K<sub>i</sub> values of diazepam-sensitive and diazepam-insensitive [3H]Ro 15-4513-binding sites were in the range of 30–100 nM and >60,000 nM, respectively. The data represent the mean ± S.D. of three to six independent experiments.

**Table III**

Summary of the estimated abundance of diazepam-sensitive GABA<sub>A</sub> receptors containing different types of α subunits in whole mouse brain membranes

| Subunit combination | Total diazepam-sensitive receptor population | %     |
|---------------------|---------------------------------------------|-------|
| α<sub>1</sub>α<sub>1</sub> |                                            | 61    |
| α<sub>2</sub>α<sub>2</sub> |                                            | 12    |
| α<sub>3</sub>α<sub>3</sub> |                                            | 4     |
| α<sub>1</sub>α<sub>2</sub> |                                            | 13    |
| α<sub>1</sub>α<sub>3</sub> |                                            | 15    |
| α<sub>2</sub>α<sub>3</sub> |                                            | 2     |

The abundance of various receptor subpopulations was calculated from the data presented in Table I. The increase in diazepam-insensitive binding in the single-mutant mice corresponds to the abundance of the receptor populations containing equal α subunits because only receptors containing two mutated α subunits are rendered insensitive to diazepam binding. The receptor populations containing two heterologous α subunits were calculated by subtracting the sum of diazepam-insensitive binding sites present in the respective single-mutant mice from diazepam-insensitive binding sites present in the corresponding double-mutant mice. The difference in the sum of diazepam-insensitive sites present in the single-mutant mice and the diazepam-insensitive sites in the corresponding double-mutant mice represents the amount of the respective receptors containing two different α subunits. For example, the abundance of α<sub>1</sub>α<sub>2</sub> receptors is calculated as follows: 86% (diazepam-insensitive binding in α<sub>1</sub>H101R α<sub>2</sub>H101R mice) minus 73% (the sum of diazepam-insensitive binding in α<sub>1</sub>H101R mice (61%) and α<sub>2</sub>H101R mice (12%)) equals 13%.

**Hint**

The presence of diazepam-sensitive and diazepam-insensitive binding sites in brain preparations permitted the estimation of the size of GABA<sub>A</sub> receptor populations in brain containing the different α subunit combinations (Table I). This analysis is restricted to the diazepam-sensitive α<sub>1</sub>, α<sub>2</sub>, and α<sub>3</sub> containing receptors expressed in brain, which, however, represent the vast majority of GABA<sub>A</sub> receptors (summarized in Table III). 2) Data on the presence of diazepam-sensitive and diazepam-insensitive binding sites in immuno-isolated GABA<sub>A</sub> receptors allowed an estimate on the abundance of the different α subunit combinations present within a particular GABA<sub>A</sub> receptor population (e.g. the α<sub>1</sub>α<sub>3</sub> receptor combination in α<sub>1</sub>-containing receptors; Table II). The data obtained from this analysis are summarized in Table IV. Because the experimental data exhibit partially considerable standard deviation (Table II), the data are considered to represent a rough estimate of the receptor populations.

With about 61%, the α<sub>1</sub>α<sub>1</sub>-containing receptor is by far the most abundant GABA<sub>A</sub> receptor subtype in brain, whereas α<sub>2</sub>α<sub>2</sub>- (12%) and α<sub>3</sub>α<sub>3</sub>-containing receptors (4%) are considerably less expressed (Table III). Interestingly, levels of α<sub>1</sub>α<sub>2</sub>-containing receptors (13%) are similar to that of α<sub>2</sub>α<sub>2</sub>-containing receptors and levels of α<sub>1</sub>α<sub>3</sub>-containing receptors (15%) are considerably higher than that of α<sub>3</sub>α<sub>3</sub>-containing receptors (4%; Table III). However, receptors containing the α<sub>2</sub> and α<sub>3</sub> subunit in a single receptor complex appear to be rarely expressed in relation to the total diazepam-sensitive GABA<sub>A</sub> receptor population (2%; Table III). Corresponingly, a similar distribution of the prevalence of
the various α subunit combinations was detected within the α1-, α2-, and α3-containing receptor populations (Table IV). Again, the α1α1 combination (84%) predominated the α1α2 (7%) and α1α3 combinations (8%) in the α1-containing receptor population. Within the α2 receptor population the α2α2 (46%) and the α1α2 combination (36%) are expressed to a similar extent, whereas the α2α3 combination (19%) is less abundant. Finally, within the α3-containing receptor population, the α2α3 (19%) and α3α3 combinations (27%) are clearly dominated by the α1α3 combination (56%). These values are in good agreement with the data derived from the immunodepletion experiments (Table IV). In addition, the data fit nicely to values published so far on the abundance of the α2α2 combination in the α2-containing receptor population of rat hippocampus and cerebral cortex (36% versus 36 and 39%, respectively) (6, 8) and the α1α3 combination in the α3-containing receptor population in the cerebral cortex (56% versus 55%) (6, 8). However, for the α1α3 combination in the α1-containing receptor population in cerebral cortex, an abundance of 24% was reported (6, 8), whereas the estimate of the present report yielded a value of 8% for whole mouse brain extracts. It is currently unclear whether this mismatch is due to experimental reasons, the brain areas used (cerebral cortex versus whole brain), or the species analyzed (rat versus mouse).

Interestingly, only in the α1-containing receptor population the α1α1 combination is the most prevalent one, whereas in the α2 and particularly in the α3 receptor population receptors with two different types of α subunits predominate. Because GABA_A receptors containing two different types of α subunits appear to be expressed at considerable levels, it is conceivable that the inclusion of a second type of a subunit may influence GABA_A receptor function. However, the consequences of two different types of α subunits within a single receptor complex on GABA_A receptor function are largely unknown. In particular, it is not clear how the expression of heterologous α subunits in a receptor complex affects functional benzodiazepine receptor pharmacology. So far, electrophysiological experiments on recombinant α1δβ2γ2, α1δβ2γ2, and α1δβ2γ2 indicate that receptors containing two different α subunit variants display unique kinetics and pharmacological properties (23–26). All of these studies inherently lack the ultimate certainty that indeed predominantly the subunit combination containing two heterologous α subunits was measured and not a mixture of different subunit combinations containing only a single α subunit variant. However, recent successful approaches linking the subunits covalently together to assess the subunit arrangement within the receptor complex and the functional consequences of subunit position in the receptor pentamer provide now the basis for analyzing GABA_A receptor subtypes of predefined subunit arrangement (27–29). A first study on the functional consequences of the position of α1 and α6 subunits in the same receptor pentamer clearly demonstrated that only receptors with the α1 subunit next to the γ2 subunit (γ2β-α6-δβ-α1) displayed modulation by diazepam, whereas receptors with the α6 subunit next to the γ2 subunit (γ2-β-α1-β-α6) were insensitive to diazepam (28). These data support the results of the present study indicating that only receptors containing two mutated α subunits are insensitive to diazepam binding. In addition, the data suggest that the nonmutated, diazepam-sensitive α subunit preferentially assembles with the γ2 subunit.

In conclusion, the results of this study indicate that the mouse lines containing the α1'H21 mutation represent a valuable model for analyzing the abundance of receptor populations containing two different types of α subunits. This approach allows the analysis to be performed on membrane fractions, tissue homogenates, or even tissue sections without solubilization and immunoprecipitation of the receptors. A detailed analysis of receptor subtypes containing two different types of α subunits on a brain regional level by quantitative receptor autoradiography on brain sections is now feasible.

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