Published in final edited form as:

Curr Protoc Pharmacol. ; 63: 1.7.1–1.7.20. doi:10.1002/0471141755.ph0107s63.

Characterization of GABA Receptors

S.J. Enna and Kenneth E. McCarson

University of Kansas Medical Center, Kansas City, Kansas

Abstract

Described in this unit are ligand binding assays for GABA_A, GABA_B, and the homomeric ρ GABA_A (formerly GABA_C) receptor recognition sites in brain tissue. Although GABA binding sites are present in peripheral organs, most research is directed toward examining these receptors in the CNS. These assays may also be used to determine the affinity of an unlabeled compound for the GABA binding sites. Excluded from the unit are ligand binding assays for other components of the GABA_A receptor complex, such as the benzodiazepine (Unit 1.16) or ion channel (Unit 1.18) binding sites.

INTRODUCTION

γ -Aminobutyric acid (GABA) is an inhibitory amino acid neurotransmitter that is widely distributed throughout the central nervous system (CNS). While there is ample evidence indicating the existence of a variety of molecularly and pharmacologically distinct receptors for this substance, for the purpose of ligand binding assays, they are currently divided into three broad categories: GABA_A, GABA_B, and homomeric ρ subunit GABA_A site, formally referred to as the GABA_C receptor (Table 1.7.1).

While [³H]GABA was used initially to label the three types of GABA receptors, attempts have been made to develop radioligands selective for each. [³H]Muscimol, a highly potent and selective GABA_A receptor agonist, is the ligand of choice for labeling this receptor (see Basic Protocol 1 and see Alternate Protocol 1). [³H]GABA is still the preferred ligand for labeling GABA_B sites, since it yields the most consistent and robust data (see Basic Protocol 2). However, [³H]baclofen, a selective agonist for the GABA_B site, has also been used for binding (see Alternate Protocol 2), as has [³H]CGP-54626, a GABA_B receptor antagonist (Bittiger et al., 1993). As for homomeric ρ subunit GABA_A site (GABA_C), [³H]GABA is the only commercially available radioligand for this site (see Basic Protocol 2), although binding with [³H]*cis*-4-aminocrotonic acid has been reported (Drew and Johnston, 1992).

Given the large number of GABA receptors in brain tissue, care must be taken when utilizing [³H]GABA as the labeling ligand to ensure the displaceable (specific) binding represents attachment only to the receptor of interest. This is accomplished by using particular brain regions or tissues, modifying the tissue preparation or incubation buffer, and including unlabeled substances in the assay to prevent attachment of the radioligand to other GABA binding sites.

Update

In recent years research has focused more on identifying and developing allosteric, rather than orthosteric, GABA receptor agonists and antagonists. Inasmuch as the protocols detailed in this unit describe assays aimed at characterizing interactions at the orthosteric GABA receptor recognition site, they remain current. The ligand of choice for labeling the GABA_A site is still radiolabeled muscimol, although [³H]GABA continues to be employed as well. For GABA_B receptor binding assays, the agonists [³H]baclofen and [³H]GABA remain popular as radioligands. An assay employing [³H]CGP-54626 (American Custom

Chemicals Corp., San Diego, CA) a selective, high affinity, GABA_B receptor antagonist has been developed for labeling this site as well (Bittiger et al., 1993). This antagonist ligand displays the following K_c and B_{max} values: rat brain, 2.3 nM and 1.1 pmol/mg protein; human recombinant, ~3.6 nM and ~13 pmol/mg protein. The GABA binding site formerly designated as GABA_C is now recognized as a homomeric ρ subunit GABA_A site, rendering the GABA_C designation obsolete (<http://www.iuphar-db.org/DATABASE/FamilyMenuForward?familyID=72>). As with most binding assays, all of these are now routinely conducted using microplates containing 96, but sometimes 384 or more, sample wells.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL 1

MEASUREMENT OF GABA_A RECEPTOR BINDING IN RAT BRAIN MEMBRANES USING [³H]MUSCIMOL

Described in this protocol is an in vitro assay for labeling the GABA_A receptor in rat brain membranes using [³H]muscimol. Unlabeled bicuculline methiodide or unlabeled GABA is used to define nondisplaceable binding (blank), which when subtracted from total binding (assays containing tissue and [³H]muscimol alone) reveals the amount of specific radioligand binding to the GABA_A receptor. This protocol may also be used to determine the affinity of the GABA_A binding site for an unlabeled compound. For a general screen, [³H]muscimol binding is challenged with some fixed concentration (10 or 50 μ M) of the test agents. To calculate a K_i for an unlabeled compound that competes with [³H]muscimol for the GABA_A binding site, eight to ten concentrations of the test agent should range from at least 10-fold below to 10-fold above its estimated IC₅₀ value.

Materials

Frozen membrane preparation (see Support Protocol)

50 mM Tris citrate buffer (pH 7.1 at 4°C; adjust pH of 1 M Tris base with a concentrated solution of citric acid at 4°C, then dilute 1:20)

[³H]Muscimol (5 to 20 Ci/mmol; PerkinElmer NEN)

(–)-Bicuculline methiodide (Sigma-Aldrich, or ICN Biomedicals)

Muscimol (unlabeled)

γ -Amino-*n*-butyric acid (GABA; Sigma-Aldrich, or ICN Biomedicals)

Test compound: unlabeled competitor (optional)

Scintillation cocktail

50-ml polypropylene centrifuge tubes

Tissue homogenizer (e.g., Polytron, Brinkmann; Tissumizer, Tekmar)

Refrigerated centrifuge (Sorvall RC-5 with SS-34 and SM-24 rotors, or equivalent)

13 × 100-mm borosilicate glass Pyrex culture tubes

Brandel cell harvester

Whatman GF/B glass fiber filters

Liquid scintillation counter and vials

NOTE: Maintain tissue samples in an ice-water bath ($\sim 4^{\circ}\text{C}$) throughout the preparation and incubation procedures. Be sure to adjust buffer to the proper pH at 4°C , as the pH of Tris buffers varies significantly with temperature.

Prepare receptors for binding assay

1. In 50-ml polypropylene centrifuge tubes, resuspend previously frozen tissue or brain membranes in 50 vol ice-cold Tris citrate buffer using the tissue homogenizer (midpoint setting for ~ 30 sec).
2. Centrifuge homogenate 10 min at $50,000 \times g$, 4°C .
3. Discard the supernatant and repeat tissue resuspension and centrifugation five additional times as in steps 1 and 2.

Multiple washings of the tissue are necessary to remove endogenous GABA that is present in high concentrations in brain. Residual GABA in the membrane preparation interferes with the attachment of the radioligand to the binding site.

4. Resuspend the final pellet with the tissue homogenizer in sufficient buffer to yield a protein concentration of ~ 0.5 mg protein/ml.

Protein concentration may be measured using Bradford, BCA (Pierce), Lowry, or other suitable assay (see APPENDIX 3A) with BSA as reference standard.

With this protocol, [^3H]muscimol binding is linear up to ~ 1.0 mg protein/ml. As with all radioligand binding assays, it is important to conduct the incubation at a tissue concentration within the linear portion of the binding assay.

Measure binding of [^3H]muscimol to GABA_A receptor

- 5a** *For competition assays:* In separate 13×100 -mm borosilicate glass culture tubes on ice, assemble the following components in 1-ml volumes, diluted with Tris citrate buffer:

4.0 nM [^3H]muscimol (to measure total binding);

4.0 nM [^3H]muscimol + [200 μM (–)-bicuculline methiodide or 200 μM GABA] (to define nondisplaceable binding);

4.0 nM [^3H]muscimol + various concentrations of unlabeled competitor (test compound).

Perform all assays in duplicate or triplicate.

The concentrations of [^3H]muscimol, unlabeled bicuculline methiodide, or unlabeled GABA in the final 2-ml incubation volume will be 2.0 nM, 100 μM , and 100 μM , respectively.

- 5b** *To generate binding-site saturation data by radioligand displacement:* Prepare 1-ml solutions in tubes as described in step 5a but containing:

4.0 nM [^3H]muscimol;

4.0 nM [^3H]muscimol + various concentrations (1 nM to 1 μM) of unlabeled muscimol.

Saturation data may also be obtained using increasing concentrations of the radioligand in the presence (blank) and absence (total binding) of a saturating concentration (200 μM) of unlabeled muscimol or GABA (Fig.

1.7.1). *Cost considerations normally preclude this type of analysis, as it is impractical to use concentrations of radioligand sufficient to saturate low-affinity sites.*

- 6 To begin the assays, add 1-ml portions of tissue suspension to the tubes and gently vortex to mix the contents.
- 7 Incubate 30 min in an ice-water bath (4°C) to achieve binding equilibrium.
The final tissue concentration in the 2-ml incubation medium is ~0.25 mg protein/ml.
- 8 Terminate the binding reaction by filtering the contents of the test tube through glass-fiber filters maintained under reduced pressure in the cell harvester.
- 9 Rapidly (3 to 5 sec each) wash each filter three times with 3 ml ice-cold Tris citrate buffer.
- 10 Transfer each filter to a scintillation vial containing 4 ml scintillation cocktail.
- 11 Shake vigorously for 30 min at room temperature, then place the vials in the liquid scintillation counter and quantify radioactivity.

Instead of shaking, the vials may be vortexed briefly and allowed to stand overnight at room temperature before being placed into the scintillation counter. The amount of time necessary for the radioactivity to be leached from the filter by the scintillation cocktail should be determined empirically by quantifying radioactivity at various times after addition of the scintillation cocktail until the number of counts becomes stable.

Analyze binding data

- 12 Convert cpm data to [³H]muscimol bound and plot against added [³H]muscimol (see Fig. 1.7.1A).
- 13 Estimate the dissociation constant (K_d) and maximum binding values (B_{max}) for the assay using a Scatchard plot of bound ligand versus bound/free ligand (see Fig. 1.7.1B). For a more precise analysis of binding data it is best to use a program designed for this purpose, such as LIGAND (Munson and Rodbard, 1980) or Prism (GraphPad, San Diego, CA).

UNIT 1.3 provides details on determining these values from concentration-response curves. Table 1.7.2 lists IC_{50} values for competitors of GABA_A binding determined by displacement of [³H]muscimol.

ALTERNATE PROTOCOL 2

MEASUREMENT OF GABA_A RECEPTOR BINDING IN RAT BRAIN MEMBRANES USING [³H]GABA

While the [³H]muscimol binding assays has a high degree of specificity, the radioligand can be quite expensive. An alternative presented below is to use the less expensive, but also less selective, [³H]GABA to determine binding to GABA_A receptors. Selectivity is increased by treating the membranes with detergent. Incubation with Triton X-100 and multiple resuspensions and centrifugations destroys neuronal GABA uptake sites that may bind [³H]GABA and rids the tissue of endogenous GABA, which competes for binding sites with the radioligand. Detergent treatment is less important when [³H]muscimol is used to label GABA_A receptors because muscimol has a low affinity for the GABA transporter (Krogsgaard-Larsen et al., 1983).

Additional Materials (also see Basic Protocol 1)

- 10% Triton X-100 in Tris citrate buffer (see Basic Protocol 1 for buffer)
- [³H]GABA (PerkinElmer NEN)
- Tissue solubilizer (e.g., BTS-450, Beckman, or equivalent)
- Scintillation cocktail compatible with organic solvents
- 15-ml polypropylene centrifuge tubes

Prepare GABA_A receptors

1. In 50-ml polypropylene centrifuge tubes, resuspend previously frozen tissue or brain membranes in 50 vol ice-cold Tris citrate buffer using the tissue homogenizer (midpoint setting for ~30 sec).
2. Centrifuge homogenate 10 min at 50,000 × g, 4°C.
3. Resuspend the resultant pellet in sufficient Tris citrate buffer to yield a concentration of 1 mg protein/ml.

Protein concentration may be measured using Bradford, BCA (Pierce), Lowry, or other suitable assay (see APPENDIX 3A) with BSA as reference standard.

4. Add to the tissue suspension sufficient 10% Triton X-100 in Tris citrate buffer to yield a 0.05% (v/v) concentration of detergent in the suspension.
5. Incubate 20 min in a 37°C water bath.
6. Centrifuge the tissue suspension 10 min at 50,000 × g, 4°C.
7. Resuspend and centrifuge the tissue two additional times as in steps 1 and 2.
8. Using the tissue homogenizer, resuspend the pellet in sufficient Tris citrate buffer to yield a final concentration of ~0.5 mg protein/ml.

Measure [³H]GABA binding to GABA_A receptors

- 9a** *For competition assays:* In separate 15-ml polypropylene tubes on ice, assemble the following components in 1-ml volumes, diluted with Tris citrate buffer:

8.0 nM [³H]GABA (to determine total binding);

8.0 nM [³H]GABA + [200 μM (–)-bicuculline methiodide or 20 μM muscimol] (to determine nondisplaceable binding);

8.0 nM [³H]GABA + various concentrations of unlabeled competitor (test compound).

Perform all assays in duplicate or triplicate.

Final concentrations of [³H]GABA, bicuculline methiodide, and muscimol in the final 2-ml incubation volume will be 4 nM, 100 μM, and 10 μM, respectively. The unlabeled bicuculline methiodide or unlabeled muscimol is used to define nonspecific binding (blank) which, when subtracted from total binding (tissue in tubes containing [³H]GABA alone), reveals the amount of radioligand bound to the GABA_A receptor.

- 9b** *To generate binding site saturation data by displacement:* Prepare 1-ml solutions in tubes as described in step 9a but containing:

8.0 nM [³H]GABA;

8.0 nM [^3H]GABA + various concentrations (2.0 to 1000 nM) of unlabeled GABA.

As described in Basic Protocol 1 with [^3H]muscimol, this assay may be used as a general screen for assessing the affinity of unlabeled compounds for the GABA_A receptor binding site.

- 10 To begin the assays, add 1 ml of the tissue suspension to each of the chilled tubes. Gently vortex each tube to mix the contents.

The final tissue concentration in the 2-ml incubation volume will be ~0.25 mg protein/ml, which is within the tissue linearity range for [^3H]GABA binding to GABA_A receptors.

- 11 Incubate 5 min in an ice-water bath (4°C) to achieve binding equilibrium.

- 12 Terminate the binding reaction by centrifuging the mixture 10 min at 50,000 × g, 4°C.

To accurately measure the low-affinity GABA binding site, centrifugation rather than filtration is used to terminate the [^3H]GABA binding assay to minimize loss of bound radioligand during the more thorough rinsing procedure associated with filtration. Since, with the Triton wash, the K_d for high-affinity [^3H]GABA binding is ~20 nM or less, the filtration procedure can be used with this radioligand if the higher-affinity site is the primary target.

- 13 Discard the radioactive supernatant, then rinse the tissue pellets rapidly and superficially three times with 5 ml ice-cold Tris citrate buffer.

Caution must be exercised to ensure the tissue pellets, or portions of them, are not dislodged from the bottom of the tubes during the rinse procedure. The buffer should be sprayed against the wall of the tube opposite the tissue so the pellet is not exposed to the full force of the fluid.

- 14 Gently dry the inside of each tube with tissue paper or cotton swabs to remove any residual rinse buffer, taking care not to touch the pellet.

- 15 Place 1 ml tissue solubilizer into each tube, ensuring that the pellet is submerged.

- 16 Allow tissue to dissolve in solubilizer at room temperature, or by incubating the tubes in a 37°C water bath.

- 17 Once the tissue is dissolved, add 4 ml organic solvent-compatible scintillation cocktail.

The tissue solubilizer contains toluene.

- 18 Transfer the contents of each tube into individual scintillation vials, then quantify radioactivity using liquid scintillation spectrometry.

- 19 Perform data analysis using an appropriate binding assay program, such as LIGAND or Prism.

UNIT 1.3 provides details on plotting and analyzing concentration-response curves. Sample results obtained for [^3H]GABA binding to GABA_A receptors in rat brain tissue are shown in Figure 1.7.2. Table 1.7.2 lists IC₅₀ values determined for competitors of GABA_A binding determined by displacement of [^3H]GABA.

BASIC PROTOCOL 2

MEASUREMENT OF GABA_B RECEPTOR BINDING IN RAT BRAIN MEMBRANES USING [³H]GABA

Detailed in this protocol is an in vitro assay for labeling GABA_B receptors in rat brain membranes using [³H]GABA as the labeling ligand. Incubation with Triton X-100 and multiple resuspensions and centrifugations destroy neuronal GABA uptake sites that may bind [³H]GABA and rid the tissue of endogenous GABA, which competes for the binding site with the radioligand. There is an absolute requirement for calcium in the incubation medium for [³H]GABA to attach preferentially to the GABA_B receptor. In addition, isoguvacine is used as a selective GABA_A receptor agonist, which is added in excess to prevent attachment of [³H]GABA to this site.

Materials

Frozen membrane preparation (see Support Protocol)

50 mM Tris·Cl (pH 7.4 at 25°C; *APPENDIX 2A*)/2.5 mM CaCl₂

Triton X-100

0.05 M Tris citrate buffer (pH 7.1 at 4°C; adjust pH of 1 M Tris base with a concentrated solution of citric acid at 4°C, then dilute 1:20)

Isoguvacine (Sigma-Aldrich, or ICN Biomedicals)

[³H]γ-Amino-*n*-butyric acid (GABA; 25 to 40 Ci/mmol; PerkinElmer NEN)

(±)-Baclofen or GABA (unlabeled; Sigma-Aldrich, or ICN Biomedicals)

Test compound: unlabeled competitor (optional)

Tissue solubilizer (e.g., BTS-450, Beckman, or equivalent)

Scintillation cocktail compatible with organic solvents

50- and 15-ml polypropylene centrifuge tubes

Tissue homogenizer (e.g., Polytron, Brinkmann; Tissumizer, Tekmar)

Refrigerated centrifuge (Sorvall RC-5 with SS-34 or SM-24 rotors, or equivalent)

37° and 25°C water baths

Liquid scintillation counter and vials

NOTE: Be sure to adjust buffers to the proper pH at the temperatures indicated, as the pH of Tris buffers varies significantly with temperature.

Prepare GABA_B receptors

1. In 50-ml polypropylene centrifuge tubes, resuspend frozen tissue or brain membranes in 100 vol Tris·Cl/2.5 mM CaCl₂ using the tissue homogenizer (midpoint setting for 30 sec).
2. Centrifuge the homogenate 10 min at 1000 × *g*, 4°C.
3. Pour the supernatant into a fresh 50-ml polypropylene centrifuge tube.
4. Add sufficient Triton X-100 diluted in Tris citrate buffer (see Alternate Protocol 1, step 1) to yield a final concentration of 0.03% (v/v).
5. Incubate the supernatant 30 min in the 37°C water bath.

6. Centrifuge the supernatant 10 min at $50,000 \times g$, 4°C .
7. Resuspend the resultant tissue pellet with the tissue homogenizer in the same volume of buffer as in step 1, then centrifuge the homogenate 10 min at $50,000 \times g$, 4°C .
8. Repeat step 7.
9. Resuspend the pellet with the tissue homogenizer in sufficient buffer to yield a final concentration of ~ 1 mg protein/ml.

Protein concentration may be measured using Bradford, BCA (Pierce), Lowry, or other suitable assay (see APPENDIX 3A) with BSA as reference standard.

Measure [^3H]GABA binding to GABA_B receptors

- 10a** *For competition assays:* In separate 15-ml polypropylene centrifuge tubes on ice, assemble the following components in a small volume (10 to 20 μl) and dilute to 100 μl with Tris-Cl/2.5 mM CaCl:

100 nM [^3H]GABA + 400 μM isoguvacine (to determine total binding);

100 nM [^3H]GABA + 400 μM isoguvacine + [1 mM ($\pm$)-baclofen *or* 1 mM unlabeled GABA] (to determine nondisplaceable binding);

100 nM [^3H]GABA + 400 μM isoguvacine + various concentrations of unlabeled competitor (test compound).

Perform all assays in duplicate or triplicate.

Final concentrations in the 1-ml incubation volume will be 40 μM isoguvacine and 10 nM [^3H]GABA.

The unlabeled ($\pm$)-baclofen or unlabeled GABA is used to define nondisplaceable binding (blank), which when subtracted from total binding (in tubes containing only [^3H]GABA and isoguvacine) reveals the amount of specific binding to the GABA_B receptor.

- 10b** *To generate binding site saturation data by radioligand displacement:* Prepare 100- μl solutions in tubes as described in step 10a but containing the following:

100 nM [^3H]GABA + 400 μM isoguvacine;

100 nM [^3H]GABA + 400 μM isoguvacine + various concentrations of unlabeled GABA (0.1 to 100 μM).

The high-affinity GABA_B binding site may also be characterized using increasing concentrations of [^3H]GABA in the presence and absence of a saturating (100- μM) concentration of unlabeled GABA (Fig. 1.7.3).

As described in step 5b of Basic Protocol for [^3H]muscimol binding to GABA_A receptors, this assay may be used as a general screen for assessing the affinity of unlabeled compounds for the GABA_B receptor binding site. Table 1.7.3 lists IC_{50} values for competitors of GABA_B substrates determined by displacement of [^3H]GABA.

- 11** Add 900 μl of the tissue suspension to each tube and gently vortex to mix the contents.

The tissue concentration in the incubation medium will be slightly less than 1.0 mg protein/ml, which is within the tissue linearity range for [^3H]GABA binding to GABA_B receptors (Bowery et al., 1985).

- 12 Incubate the mixture 10 min at 25°C to achieve binding equilibrium.
- 13 Terminate the binding reaction by centrifuging 10 min at $50,000 \times g$, 4°C.
- 14 Discard the supernatant, then rinse the tissue pellets rapidly (3 to 5 sec) and superficially three times with 5 ml ice-cold Tris/CaCl₂ buffer.
Caution must be exercised to ensure the tissue pellets, or portions of them, are not dislodged from the bottom of the tube during the rinsing procedure. The buffer should be sprayed against the wall of the tube opposite to the tissue so that the pellet is not exposed to the full force of the fluid.
- 15 Gently dry the inside of each tube with tissue to remove any residual rinse buffer, taking care not to touch the tissue pellet.
- 16 Place 1 ml of tissue solubilizer into each tube, ensuring that the pellet is submerged.
- 17 Allow tissue to dissolve in the solubilizer at room temperature or by incubating the tubes in a 37°C water bath.
- 18 Once the tissue is dissolved, add 4 ml organic solvent-compatible scintillation cocktail.
The tissue solubilizer contains toluene.
- 19 Transfer the contents of each tube into individual liquid scintillation counting vials, then quantify radioactivity using liquid scintillation spectrometry.
- 20 Perform data analysis using an appropriate binding assay program, such as LIGAND or Prism.

UNIT 1.3 provides details on plotting and analyzing concentration-response curves. Sample results obtained for [³H]GABA binding to GABA_B receptors in rat brain tissue are shown in Figure 1.7.3.

ALTERNATE PROTOCOL 2

MEASUREMENT OF GABA_B RECEPTOR BINDING IN RAT BRAIN MEMBRANES USING [³H]BACLOFEN

Baclofen, a selective agonist for the GABA_B site, may be used as a radioligand for this receptor instead of GABA. Although it should be more selective than GABA for this site, it does not yield as consistent or robust data. Shown on Table 1.7.3 are the IC₅₀ values for competitors of GABA_B binding determined by displacement of [³H]baclofen.

Additional Materials (also see Basic Protocol 2)—[³H](–)-Baclofen (30 to 50 Ci/mmol; PerkinElmer NEN)

[³H](–)-Baclofen binding assay for GABA_B receptors

1. In 50-ml polypropylene centrifuge tubes, resuspend previously frozen whole tissue or brain membranes in 100 vol Tris·Cl/2.5 mM CaCl₂ using the tissue homogenizer (midpoint setting for 30 sec).
2. Centrifuge homogenate 20 min at $20,000 \times g$, 4°C.
3. Repeat steps 1 and 2 with the tissue pellet three additional times.

A thorough washing of the tissue is essential to rid it of endogenous GABA, which competes with the binding of the radioligand.

4. Resuspend the pellet with the tissue homogenizer in sufficient buffer to yield a final concentration of ~1 mg protein/ml.

Protein concentration may be measured using Bradford, BCA (Pierce), Lowry, or other suitable assay (see APPENDIX 3A) with BSA as the reference standard.

5. Measure binding to receptors and perform competition assays: see Basic Protocol 2 (GABA_B receptor binding), steps 10 to 20, and follow the procedure described, except using 50 nM [³H](–)-baclofen in place of 100 nM [³H]GABA.

The final concentration of in [³H](–)-baclofen the 1-ml assay is 5 nM.

Analyze data using an appropriate binding assay program, such as LIGAND or Prism.

Sample results obtained for [³H](–)-baclofen binding to GABA_B receptors in rat brain tissue are shown in Figure 1.7.4.

BASIC PROTOCOL 3

MEASUREMENT OF HOMOMERIC ρ SUBUNIT GABA_A (FORMERLY GABA_C) RECEPTOR BINDING IN RAT BRAIN MEMBRANES USING [³H]GABA

Described in this protocol is an in vitro assay for labeling the homomeric ρ subunit GABA_A receptor, in rat brain membranes using [³H]GABA (Drew and Johnston, 1992). As these receptors are most enriched in retina and cerebellum, the latter is the tissue of choice for this assay. While an assay utilizing [³H]*cis*-4-aminocrotic acid as the labeling ligand for this site has been published (Drew and Johnston, 1992), it is not described here because the radioligand is not available commercially and it is highly toxic. As described in Basic Protocol 1 for [³H]muscimol binding to GABA_A receptors, this assay may be used as a general screen for assessing the affinity of unlabeled compounds for this homomeric GABA_A receptor binding site.

Materials

Frozen cerebellar membrane preparation (see Support Protocol)

50 mM Tris-Cl (pH 7.4 at 20°C; APPENDIX 2A)

Isoguvacine (Sigma-Aldrich, or ICN Biomedicals)

[³H]γ-Amino-*n*-butyric acid (GABA; 25 to 40 Ci/mmol; PerkinElmer NEN)

GABA (unlabeled; Sigma-Aldrich, or ICN Biomedicals)

Test compound: unlabeled competitor (optional)

Scintillation fluid compatible with organic solvents

50-ml polypropylene centrifuge tubes

20°C shaking water bath

Tissue homogenizer (Polytron, Brinkmann; Tissumizer, Tekmar)

Refrigerated centrifuge (Sorvall RC-5 with SS-34 and SM-24 rotors, or equivalent)

Liquid scintillation counter and vials

NOTE: Be sure to adjust buffer to the proper pH at 20°C, as the pH of Tris buffers varies significantly with temperature.

Prepare homomeric ρ subunit GABA_A receptors

1. In 50-ml polypropylene centrifuge tubes, resuspend cerebellar membranes in sufficient 50 mM Tris-Cl to yield a final concentration of ~8.0 mg protein/ml using the tissue homogenizer (midpoint setting for ~30 sec).

Protein concentration may be measured using Bradford, BCA (Pierce), Lowry, or other suitable assay (see APPENDIX 3A) with BSA as reference standard.

2. Incubate the tissue suspension 45 min at 20°C in a shaking water bath.
3. Centrifuge the tissue suspension 10 min at 8000 × g, 4°C.
4. Resuspend the resultant pellet using the tissue homogenizer in the same volume of buffer as in step 1.
5. Incubate the tissue suspension 15 min in the 20°C shaking water bath.
6. Centrifuge the tissue suspension 10 min at 8000 × g, 4°C.
7. Repeat steps 4 to 7 two additional times.

Multiple resuspensions and centrifugations rid the tissue of endogenous GABA, which competes with [³H]GABA for attachment to this homomeric GABA_A receptor binding site.

8. Resuspend the final tissue pellet in sufficient buffer containing 40 μM isoguvacine to yield a tissue concentration of ~3 mg protein/ml.
9. Allow suspension to stand 10 min at room temperature before initiating the binding assay.

The 10-min delay allows sufficient time for the isoguvacine to block other GABA_A receptors.

Measure [³H]GABA binding to GABA_C receptors

- 10a** *For competition assays:* In separate 1.5-ml microcentrifuge tubes on ice, assemble the following components in a 900-μl volume, diluted with 50 mM Tris-Cl, pH 7.4 (but calculating the concentrations for a 1000-μl final volume):

5 nM [³H]GABA + 40 μM isoguvacine (to determine total binding);

5 nM [³H]GABA + 40 μM isoguvacine + 300 μM unlabeled GABA (to determine nondisplaceable binding);

5 nM [³H]GABA + 40 μM isoguvacine + various concentrations of unlabeled competitor (test compound).

Perform all assays in duplicate or triplicate.

As isoguvacine is a GABA_A receptor agonist having a low affinity for the homomeric ρ subunit site, it is added in excess to prevent binding of [³H]GABA to other GABA_A receptors. The unlabeled GABA is used to define nondisplaceable binding (blank) which, when subtracted from total binding (tissue in tubes containing [³H]GABA and isoguvacine alone), reveals the amount of specific binding to the homomeric ρ subunit GABA_A receptor.

- 10b** *To generate binding site saturation data by ligand displacement:* Prepare 900-μl solutions in tubes as described in step 10a, but containing the following (again calculating the concentrations for a 1000-μl final volume):

40 μ M isoguvacine + 5 nM [3 H]GABA;

40 μ M isoguvacine + 5 nM [3 H]GABA + various concentrations of unlabeled GABA (5 nM to 5 μ M).

- 11 Add 100 μ l of the tissue suspension (300 μ g protein) to each tube and gently vortex to mix the contents.
The final tissue concentration in the assay medium (~300 μ g/ml) is within the linearity range for binding to receptors (Drew and Johnston, 1992).
- 12 Incubate the mixture 10 min in the 20°C shaking water bath to achieve binding equilibrium.
- 13 Terminate the binding reaction by microcentrifuging the samples 5 min at 10,000 $\times$ g, 20°C.
- 14 Rinse the pellets rapidly and superficially three times with 1.0 ml ice-cold distilled water.

Caution must be exercised to ensure the tissue pellets, or portions of them, are not dislodged from the bottom of the tube during the rinsing procedure. The ice-cold water should be added slowly to the tube, directing the spray away from the tissue sample so it is not exposed to the full force of the fluid.

- 15 Add 1 ml ice-cold distilled water to the microcentrifuge tube, submerging the pellet.
- 16 Leave samples 24 hr at room temperature.
- 17 Vortex each sample, then transfer to scintillation vials.
- 18 Add 4 ml organic solvent-compatible scintillation cocktail.
- 19 Quantify radioactivity using liquid scintillation spectrometry.
- 20 Perform data analysis using an appropriate binding assay program, such as LIGAND or Prism.

UNIT 1.3 provides details on plotting and analyzing concentration-response curves. Sample results obtained for [3 H]GABA binding to the homomeric ρ subunit GABA_A receptors in rats are shown in Fig. 1.7.5. Table 1.7.4 lists IC₅₀ values for inhibitors at this site as determined by displacement of [3 H]GABA.

SUPPORT PROTOCOL

PREPARATION OF MEMBRANES

For all five protocols, membranes can be prepared directly from whole tissue samples that have been stored frozen. Virtually any tissue can be examined in this way, although GABA receptor binding sites are not abundant outside the CNS. For GABA_A and GABA_B receptor binding at least, the receptors appear stable with tissue frozen at -80°C for up to 3 months. In some cases it is advantageous to prepare a crude synaptosomal membrane fraction from fresh brain tissue prior to freezing. A protocol for doing so is provided below.

Materials

- Fresh brain sample
- 0.32 M sucrose, ice cold
- Potter-Elvehjem glass homogenizer with Teflon pestle

Refrigerated centrifuge (Sorvall RC-5 with SS-34 and SM-24 rotors or equivalent)

Tissue homogenizer (Polytron, Brinkmann; Tissumizer, Tekmar)

Prepare the membranes

1. Place fresh brain tissue into 15 vol ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle, and homogenize.
2. Centrifuge the homogenate 10 min at $1000 \times g$, 4°C .
3. Discard the resultant pellet and centrifuge the supernatant 20 min at $20,000 \times g$, 4°C .
4. Resuspend the pellet in 20 ml ice-cold distilled water using a tissue homogenizer (midpoint setting for 30 sec).
5. Centrifuge the suspension 20 min at $8000 \times g$, 4°C .
6. Gently agitate the tube by hand to suspend the soft buffy coat surrounding the pellet into the supernatant without disturbing the pellet itself.
7. Discard the pellet and centrifuge the suspension 20 min at $48,000 \times g$, 4°C .
8. Discard the supernatant and store the pellet (crude synaptic membrane pellet) at least 18 hr at -20°C prior to use for a GABA receptor binding assay.

When prepared and stored in this way the tissue retains binding activity for at least 3 months. For assay, the pellet is thawed and homogenized in buffer as described in step 1 of each of the individual protocols (see Basic Protocols 1 to 3 and Alternate Protocols 1 and 2).

COMMENTARY

Background Information

It has been estimated that up to 30% of the neurons in the central nervous system utilize GABA as a neurotransmitter. Given its high concentration and ubiquitous distribution, GABA appears to be the predominant inhibitory neurotransmitter in the brain. Because modifications in GABAergic transmission are likely to occur in many, if not most, disorders of the central nervous system, there is a great deal of interest in discovering or designing drugs capable of selectively regulating this neurotransmitter system. A primary target for these efforts is the GABA receptor, a plasma membrane protein that mediates the action of this neurotransmitter.

Among the three generally recognized categories of GABA binding sites, the GABA_A binding site is located on a ligand-gated chloride ion channel receptor, is inhibited by bicuculline, and, in some cases, is regulated by benzodiazepines. The GABA_B receptor is a G protein-coupled heterodimer which regulates the formation of cyclic AMP, is selectively activated by baclofen, and is not inhibited by bicuculline. Like other GABA_A receptors, the homomeric ρ subunit GABA_A sites, formerly known as GABA_C receptors, are located on a ligand-gated chloride channel, but are insensitive to bicuculline and baclofen, and selectively activated by *cis*-4-aminocrotonic acid. In general, activation of GABA receptors causes hyperpolarization of the cell.

A significant contribution to this endeavor was made with the development of ligand binding assays for GABA receptors (Enna and Snyder, 1975, 1977; Bowery et al., 1985; Drew and Johnston, 1992). Besides providing a technically simple and rapid means for determining whether a chemical has any affinity for these sites, and therefore potential as a

therapeutic agent, this methodology made it possible to examine the biochemical and molecular properties of this receptor. The initial GABA receptor binding assay, which utilized [^3H]GABA as a radioligand, labels primarily the GABA_A receptor recognition site. Over the years, other GABA_A recognition site agonist and antagonist radioligands have been developed, including [^3H]muscimol, [^3H]piperidine-4-sulfonic acid, [^3H]THIP (a structural analog of muscimol), and [^3H]bicuculline (Möhler and Okada, 1977; Beaumont et al., 1978; Krogsgaard-Larsen et al., 1981; Falch and Krogsgaard-Larsen, 1982). Of these, only [^3H]muscimol and [^3H]bicuculline are currently commercially available, and given its high affinity and selectivity for GABA_A receptors, muscimol is generally preferred for binding assays.

Ligand binding assays revealed other components of the GABA_A receptor that could serve as targets for therapeutic agents, including the [^3H]α-dihydropicrotoxin binding site (Unit 1.18), a component of the GABA_A receptor-associated chloride ion channel (Ticku et al., 1978). Binding assays suggest this may be the site of action of some sedative-hypnotic agents, such as the barbiturates (Olsen, 1981).

A component of most GABA_A receptors is labeled with benzodiazepines, such as [^3H]flunitrazepam (Möhler et al., 1980) (Unit 1.16). These drugs bind to a site on the GABA_A receptor physically independent of, but associated with, the neurotransmitter recognition and ion channel binding sites. Molecular cloning studies revealed the GABA_A receptor is a pentameric structure that forms a chloride ion channel spanning the plasma membrane (Möhler et al., 1997). Eighteen subunits (six α, three β, three γ, one σ, one θ, one ε and three ρ) have been identified that, in various combinations, form physiologically active GABA_A receptors. Although the potential number of molecularly distinct GABA_A receptors is large given the number of subunits and the pentameric structure of the site, only a dozen or so are thought to be present in mammalian brain.

The predominant forms of the GABA_A receptor are those composed of α₁β₂γ₂, α₂β₃γ₂, or α₃β₃γ₂ subunits (Tan et al., 2011; Möhler et al., 2004). Binding assays suggest that GABA_A recognition site agonists, such as muscimol or GABA, attach to the β subunit of the receptor, whereas the benzodiazepine site is present only when selected α subunits are associated with certain γ subunits (Möhler et al., 1997). Given these considerations, [^3H]GABA and [^3H]muscimol are the ligands of choice for labeling the greatest number of GABA_A receptors since, by definition, all must possess a neurotransmitter receptor recognition site. In contrast, radiolabeled benzodiazepines label only those GABA_A sites that possess the correct combination of α and γ subunits. Indeed, the population of GABA_A receptors labeled may vary somewhat among the benzodiazepines, since there is a variation of affinities within this class for different combinations of α and γ subunits (Möhler et al., 1997). These findings suggest it may be possible to develop specific radioligands for each of the GABA_A receptor subtypes, facilitating the identification of more selective agonists and antagonists for these receptors.

Ligand binding assays played a major role in initially identifying GABA_B receptors (Bowery et al., 1985). While activated by GABA and baclofen, GABA_B receptors are not inhibited by bicuculline or picrotoxin, nor are they influenced by benzodiazepines. Biochemical and molecular cloning experiments indicate the GABA_B site is a heterodimeric, G protein-coupled receptor (Kaupmann et al., 1997). It would seem likely that [^3H]baclofen would serve as the radioligand of choice for labeling GABA_B receptors, as it should be more selective for this site than [^3H]GABA. However, the radiolabeled neurotransmitter itself is preferred because, for unknown reasons, it yields more robust and consistent data than [^3H]baclofen. The inclusion of calcium, as well as a GABA_A receptor agonist, in the

incubation medium helps ensure that specifically bound [^3H]GABA attaches primarily to the GABA_B site.

Recently, highly potent and selective GABA_B receptor antagonists have been used to study GABA_B binding sites (Kaupmann et al., 1997). This work resulted in the development of [^3H]CGP-54626 a selective, high affinity, GABA_B receptor antagonist that has been used for labeling this site (Bittiger et al., 1993).

Less is known about the homomeric ρ subunit GABA_A receptor, that was initially thought to be a separate class of sites that were designated as GABA_C receptors. For some time there has been evidence of bicuculline- and baclofen-insensitive [^3H]GABA binding sites (Polenzani et al., 1991; Drew and Johnston, 1992). It is now known that this receptor, which is most abundant in retina and cerebellum, is simply a homomeric GABA_A site composed solely of ρ subunits (Cutting et al., 1991), rendering the GABA_C designation obsolete. While [^3H]cis-4-aminocrotonic acid has been used as a selective ligand for this site, its toxic properties, which endanger the experimenter, preclude widespread use (Drew and Johnston, 1992).

Critical Parameters and Troubleshooting

Of the assays described in this unit, those for GABA_A are the most reliable, with a specific/nonspecific (signal/noise) binding ratio of 50% to 90%. For this assay, specific binding of either [^3H]GABA or [^3H]muscimol is most enhanced if the tissue has been treated with Triton X-100.

For both the GABA_B and homomeric ρ subunit GABA_A receptor binding assays, the specific/nonspecific ratio normally approximates 50% and is somewhat more variable than that observed with the GABA_A receptor binding assay. The reasons for this difference are unknown, although they may be related to the fact that special conditions must be used to direct radioligands away from the majority of GABA_A sites, which are abundant, and toward the GABA_B or homomeric ρ subunit GABA_A receptors.

The most common problem associated with these assays is a reduction in specific (displaceable) binding. This may occur as a result of a decrease in total binding, as a selective increase in nonspecific binding, or as a selective decrease in specific binding. In general, a decrease in the specific/nonspecific binding ratio to 40% signals a faulty assay. Detailed below are steps to be taken to address this issue (in order of priority):

1. *Prepare a fresh batch of membranes.* Most often, a decline in specific binding is due to a loss of receptors, which may be destroyed as a result of prolonged or inappropriate storage or mishandling of tissue during preparation.
2. *Terminate reaction by centrifugation rather than filtration.* The K_d values for these radioligands vary from low to mid-nanomolar. While this should be sufficient to allow for detection of specific binding with the thorough rinsing procedure associated with filtration, even a modest alteration in affinity could result in the dissociation of specifically bound ligand under these conditions. This can be rectified by terminating the reaction by centrifugation and by more gentle rinsing of the tissue, as described in Alternate Protocol 1 and in Basic Protocols 2 and 3. Comparison of results using the centrifugation and filtration methods also helps detect whether a significant amount of radioligand adheres to the glass fiber filters used in the latter, which tends to increase nonspecific binding. In general, nonspecific binding is greater with the centrifugation assay since the tissue is rinsed less thoroughly than with filtration.

3. *Prepare fresh buffer.* Preparation of a fresh stock of Tris buffer on a weekly basis is advisable, even though it is usually stable for longer periods when kept refrigerated. Nonetheless, a significant change in the amount of specific binding could be due to an error in the preparation of the buffer, such as titration to an inappropriate pH, or to microbial contamination. For the GABA_B assay, it is also important to ensure the buffer contains 2.5 mM CaCl₂, since calcium is essential for maximal binding of either [³H]GABA or [³H]baclofen to the GABA_B site. With both the GABA_B and homomeric ρ subunit GABA_A assays, the buffer must contain 40 μ M isoguvacine to prevent attachment of [³H]GABA to other GABA receptor sites. It is best to add fresh isoguvacine on a daily basis rather than include it in the stock solution of buffer.
4. *Assess purity of radioligand.* All of the radioligands used in these protocols are chemically stable if stored under the conditions recommended by the manufacturer. Thus, destruction of radioligand is seldom a problem with these assays. Nonetheless, if the tissue preparation, method of assay termination, and buffer appear fine, it is conceivable an accumulation of radioactive breakdown products could account for a change in specific binding. A simple analysis using thin-layer chromatography can be employed to assess the purity of the radioligand. The sample should be purified, or a new supply of radioligand obtained, if the purity falls below 98%.

Anticipated Results

Shown after each protocol are examples of binding site saturation data and the substrate selectivity for each site using the procedure described. Saturation data are typically analyzed using one of the programs available for this purpose, such as LIGAND or Prism. At a minimum, eight to ten, and possibly up to 24, different concentrations of the radioligand should be tested over at least a 1000-fold range to obtain reliable K_d and B_{max} values. The number of concentrations employed depends upon the number of binding sites, with 24 recommended if two sites are present. Approximate K_d and B_{max} values and the IC₅₀ data for the various assays are shown.

Time Considerations

Excluding the time required to prepare the tissue, it should be possible to conduct assays with 200 to 300 tubes on a daily basis. For each of the assays, the actual incubation period is quite brief (5 to 30 min). Most of the time is needed for preparing solutions, numbering tubes, dissolving tissue samples, and centrifugations. Although centrifugation assays require more time than filtration, 200 to 300 incubation tubes is not an unreasonable figure for an 8-hr day. Use of 96 well microplates, when possible, increases the number samples than can be analyzed in a day. This does not include quantification of radioactivity, since it may be necessary to allow samples to sit overnight to maximize counting efficiency.

Depending on whether the membranes used for assay are taken directly from a whole brain sample or a subcellular fraction, up to 3 hr may be required for preparing the samples. After preparation, the tissue samples can be divided into aliquots and stored frozen for later analysis.

Literature Cited

- Beaumont K, Chilton WS, Yamamura HI, Enna SJ. Muscimol binding in rat brain: Association with synaptic GABA receptors. *Brain Res.* 1978; 148:153–162. [PubMed: 207386]
- Bittiger H, Reymann N, Forestl W, Mickel SJ. ³H-CGP 54626: a potent antagonist radioligand for GABA_B receptors. *Pharmacol Commun.* 1993; 2:23.

- Bowery NG, Hill DR, Hudson AL. [^3H](–)-Baclofen: An improved ligand for GABA_B sites. *Neuropharmacology*. 1985; 24:207–210. [PubMed: 2986036]
- Cutting GR, Lu L, O'Hara BF, Kasch LM, Montrose-Rafizadeh C, Donovan DM, Shimada S, Antonarakis SE, Guggino WB, Uhl GR, Kazazian HH. Cloning of the γ -aminobutyric acid (GABA) ρ 1 cDNA: A GABA receptor subunit highly expressed in the retina. *Proc Natl Acad Sci U S A*. 1991; 88:2673–2677. [PubMed: 1849271]
- Drew CA, Johnston GAR. Bicuculline- and baclofen-insensitive γ -aminobutyric acid binding to rat cerebellar membranes. *J Neurochem*. 1992; 58:1087–1092. [PubMed: 1737986]
- Enna SJ, Snyder SH. Influences of ion, enzymes and detergents on γ -aminobutyric acid receptor binding in synaptic membranes of rat brain. *Mol Pharmacol*. 1977; 13:442–453. Details the effect of detergents on GABA receptor binding. [PubMed: 876033]
- Enna SJ, Snyder SH. Properties of γ -aminobutyric acid (GABA) receptor binding in rat brain synaptic membrane fractions. *Brain Res*. 1975; 100:81–97. Provides detailed description and appropriate citations for preparation of crude P₂ membrane preparation from rat brain tissue. [PubMed: 241459]
- Falch E, Krosgaard-Larsen P. The binding of the GABA agonist [^3H]THIP to rat brain synaptic membranes. *J Neurochem*. 1982; 38:1123–1129. [PubMed: 6278083]
- Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B. Expression cloning of GABA_B receptors uncovers similarity to metabotropic glutamate receptors. *Nature*. 1997; 386:239–246. [PubMed: 9069281]
- Krosgaard-Larsen P, Snowman A, Lummis SC, Olsen RW. Characterization of the binding of the GABA agonist [^3H]piperidine-4-sulfonic acid (P4S) to bovine brain synaptic membranes. *J Neurochem*. 1981; 37:401–409. [PubMed: 6267201]
- Krosgaard-Larsen, P.; Jacobsen, P.; Falch, E. Structure-activity requirements of the GABA receptor. In: Enna, SJ., editor. *The GABA Receptors*. Humana Press; Totowa, N.J: 1983. p. 149-176.
- Möhler H, Okada T. Properties of γ -aminobutyric acid receptor binding with (+)-[^3H]bicuculline methiodide in rat cerebellum. *Mol Pharmacol*. 1977; 14:256–265. [PubMed: 556312]
- Möhler, H.; Benke, D.; Benson, J.; Lüscher, B.; Rudolph, U.; Fritschy, JM. Diversity in structure, pharmacology, and regulation of GABA_A receptors. In: Enna, SJ.; Bowery, NG., editors. *The GABA Receptors*. 2. Humana Press; Totowa, N.J: 1997. p. 11-36.
- Möhler H, Battersby MK, Richards JG. Benzodiazepine receptor protein identified and visualized in brain tissue by a photoaffinity label. *Proc Natl Acad Sci U S A*. 1980; 77:1661–1670. [PubMed: 6246498]
- Möhler H, Fritschy J-M, Crestani F, Hensch T, Rudolph U. Specific GABA_A circuits in brain development and therapy. *Biochem Pharmacol*. 2004; 68:1685–1690. [PubMed: 15451412]
- Munson PJ, Rodbard D. LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem*. 1980; 107:220–239. [PubMed: 6254391]
- Olsen RW. The GABA postsynaptic membrane receptor-ionophore complex: Site of action of convulsant and anticonvulsant drugs. *Mol Cell Biochem*. 1981; 39:261–279. [PubMed: 6273709]
- Polenzani L, Woodward RM, Miledi R. Expression of mammalian γ -aminobutyric acid receptors with distinct pharmacology in *Xenopus* oocytes. *Proc Natl Acad Sci U S A*. 1991; 88:4318–4322. [PubMed: 1709741]
- Tan K, Rudolph U, Luscher C. Hooked on benzodiazepines: GABA_A receptor subtypes and addiction. *Trends Pharmacol Sci*. 2011; 34:188–197.
- Ticku MK, Ban M, Olsen RW. Binding of [^3H] α -dihydropicrotoxinin, a γ -aminobutyric acid synaptic antagonist, to rat brain membranes. *Mol Pharmacol*. 1978; 14:391–402. [PubMed: 207968]

Figure 1.7.1.

Analysis of specific [³H]muscimol binding to rat brain synaptic membranes (Beaumont et al., 1978). **(A)** Saturation of specific [³H]muscimol binding with increasing concentrations of [³H]muscimol. Rat whole brain synaptic membrane suspensions (1.0 mg protein/tube) were incubated in Tris citrate (pH 7.1) containing various concentrations of [³H]muscimol in the presence and absence of unlabeled GABA (200 μ M). **(B)** Scatchard plot of specific [³H]muscimol binding from panel A. Dissociation constant (K_d) and maximum binding (B_{max}) values for high- and low-affinity [³H]muscimol binding sites were calculated using LIGAND.

Figure 1.7.2.

Analysis of specific sodium-independent $[^3\text{H}]\text{GABA}$ binding to rat brain synaptic membranes treated with 0.05% Triton X-100 (Enna and Snyder, 1977). **(A)** Saturation of specific $[^3\text{H}]\text{GABA}$ binding with increasing concentrations of $[^3\text{H}]\text{GABA}$. **(B)** Scatchard plot of specific $[^3\text{H}]\text{GABA}$ binding from data shown in panel A. Dissociation constant (K_d) and maximum binding (B_{max}) values for high- and low-affinity $[^3\text{H}]\text{GABA}$ binding sites were calculated using LIGAND.

Figure 1.7.3.

Analysis of specific $[^3\text{H}]\text{GABA}$ binding to rat brain synaptic membranes (Bowery et al., 1985). **(A)** Saturation of specific $[^3\text{H}]\text{GABA}$ binding with increasing concentrations of $[^3\text{H}]\text{GABA}$. **(B)** Scatchard plot of specific $[^3\text{H}]\text{GABA}$ binding from panel A. Dissociation constant (K_d) and maximum binding (B_{max}) values for high- and low-affinity $[^3\text{H}]\text{GABA}$ binding sites were calculated using LIGAND.

Figure 1.7.4. Analysis of [³H](-)-baclofen binding to rat brain synaptic membranes (Bowery et al., 1985). (A) Saturation of specific [³H](-)-baclofen binding with increasing concentrations of [³H](-)-baclofen. (B) Scatchard plot of specific [³H](-)-baclofen binding from panel A. Dissociation constant (K_d) and maximum binding (B_{max}) values for high- and low-affinity [³H](-)-baclofen binding sites were calculated using LIGAND.

Figure 1.7.5.

Analysis of specific $[^3\text{H}]\text{GABA}$ binding to rat cerebellar synaptic membranes in the presence of $40 \mu\text{M}$ isoguvacine (Drew and Johnston 1992). **(A)** Saturation of specific $[^3\text{H}]\text{GABA}$ binding with increasing concentrations of $[^3\text{H}]\text{GABA}$. **(B)** Scatchard plot of specific $[^3\text{H}]\text{GABA}$ binding from panel A. Dissociation constant (K_d) and maximum binding (B_{max}) values for high- and low-affinity $[^3\text{H}]\text{GABA}$ binding sites were calculated using LIGAND.

Table 1.7.1Molecular Biology and Pharmacology of GABA Receptors^a

Receptor subtype	GenBank accession number (human clone)	Agonists	Antagonists
GABA _A	— ^b	GABA, muscimol, THIP	Bicuculline, phaclofen
GABA _{Ba}	Y10369 ^c	(-)-Baclofen, GABA,	2-Hydroxy- <i>S</i> -(<i>-</i>)-saclofen
GABA _{Bb}	Y10370 ^c	CGP 35024	
Homomeric ρ Subunit GABA _A	—	GABA, <i>cis</i> -4-aminocrotonic acid	Imidazoleacetic acid

^a Abbreviations: THIP, tetrahydroisoxazolo[5,4]pyridin-3-ol; CGP 35024, 3-aminopropyl-(*P*-methyl) phosphinic acid.

^b Ligand-gated ion channel potentially formed from $\alpha 1$ -6, $\beta 1$ -4, $\gamma 1$ -4, and δ subunits.

^c EMBL accession numbers for a and b receptors.

Table 1.7.2Activity of Ligands at GABA_A Receptors in Rat Brain Membranes

Compound ^a	IC ₅₀ (nM)	
	[³ H]Muscimol	[³ H]GABA
Muscimol	5	3
GABA	8	20
Imidazoleacetic acid	8	5
(+)-Bicuculline methiodide	2,000	5,000
(±)-Baclofen	>10,000	>10,000

^a All compounds listed are available from RBI (see *SUPPLIERS APPENDIX*).

Table 1.7.3Activity of Ligands at GABA_B Receptors in Rat Brain Membranes^a

Compound ^b	IC ₅₀ (nM)	
	[³ H](-)-Baclofen	[³ H]GABA
(-)-Baclofen	50	100
(+)-Baclofen	22,000	>100,000
GABA	22	54
Muscimol	5,000	5,000
Isoguvacine	>100,000	>100,000
Bicuculline methiodide	>100,000	>100,000

^aData based on Bowery et al. (1985).^bAll compounds listed are available from RBI (see *SUPPLIERS APPENDIX*).

Table 1.7.4

Substrate Specificity of [^3H]GABA Binding to Homomeric ρ Subunit GABA Receptors in Rat Cerebellar Membranes^a

Compound	IC ₅₀ (nM)
GABA	80
Baclofen	>100,000

^aData from Drew and Johnston (1992).