Method Title: Mouse Brain MAO-B Inhibition Assay

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Summary

This document describes an in vitro method for determining potential inhibition of the monoamine oxidase isoform B (MAO-B) in mouse brain homogenates by test drugs. The assay uses a substrate, an aminopropylether analog of methyl ester luciferin, which is converted by MAO-B to D-luciferin. The rate of D-luciferin formation is subsequently determined using the enzyme luciferase, which produces light that can be measured in a luminometer. A selective inhibitor ofMAO-B, selegiline (Deprenyl), is used as a positive control for inhibition. The use of similar assay to measure MAO-B activities in mouse liver homogenates has been reported (1), and a fluorescent assay has been used to determine MAO-B activities in mouse brain (2).

Standard reaction conditions for the mouse brain MAO-B assay are:

- Substrate concentration: 4.0 !1M
- Protein concentration: 0.2 mg/mL (10 !lg/well)
- Incubation time and temperature: 2 hours at ambient temperature
- Format: 96-well white opaque plate

Validation experiments used to select these reaction conditions are presented in Appendix 1.

Reagents and Materials

Equivalents may be used

1. MAO-Glo assay kit (Promega #V1401, enough for 200 reactions), which consists of:
   a MAO Substrate (aminopropylether analog of methyl ester luciferin)
   b Modified MAO-B Reaction Buffer- 100 mM HEPES, pH 7.5; 10% glycerol; 20% DMSO
   c Reconstitution Buffer with Esterase (converts luciferin methyl ester precursor to D-luciferin) – make sure this reagent contains esterase
   d Luciferin Detection Reagent (contains ATP and luciferase)
2. Selegiline hydrochloride (Sigma M003-250MG)
3. 96-well <u>white</u> plates (It is important to use white, not black, plates)
4. Plate luminometer capable of reading a 96-well plate (Perkin-Elmer TopCount)

Preparation of 2x Mouse Brain Homogenate

1. Collect whole brains from untreated male C57BL/6 mice and immediately immerse in ice-cold phosphate buffered saline (PBS).
2. Blot brains to remove PBS and weigh the brains. Add 10 volumes of 100 mM HEPES, pH 7.5 (giving a 1:11 homogenate <w/v>), and keep on ice.

3. Homogenize with a Polytron homogenizer, keeping the preparation ice-cold. Homogenize at a speed that gives efficient homogenization but with minimal foaming. Homogenize until the preparation has a smooth consistency.
4. Centrifuge the homogenate at 2000g for 20 minutes in a 4°C centrifuge. Collect the supernatant and discard the pellet.
5. Determine the protein concentration of the 1:11 homogenate by measuring the protein concentration of a 10-fold dilution in 100 mM HEPES buffer, pH 7.5 using the BCA protein assay (DLM-007 "BCA Protein Assay"). Dilute to 0.4 mg/mL with 100 mM HEPES buffer, pH 7.5. Aliquot the 0.4 mg/mL mouse brain homogenate into smaller 0.5 to 2.0 mL volumes, and store at -70°C until use. Homogenates should not be thawed and refrozen before use.
6. Prior to use in the MAO-B assay, thaw the homogenates on ice, pooling multiple aliquots if needed, and mix gently, avoiding foaming.

Assay Procedure

Preparation of Reagents

1. **Modified MAO-B Reaction Buffer:** (100 mM HEPES, pH 7.5, 10% glycerol, 20% DMSO)
   a. To make 250 mL of buffer, dissolve 5.96 g HEPES (MW 238.3 g/mol) in about 150 mL of water; adjust pH to 7.5.
   b. Add 25 mL of glycerol and 50 mL ofDMSO. Bring the volume to 250 mL with water. And mix by inversion. Store refrigerated for up to 1 year.
2. 4x **Substrate Stock Solution:** (16 11M substrate in MAO-B Reaction Buffer)
   a. Dissolve the lyophilized MAO Substrate in 105 11L of DMSO for the 200 reaction kit or 525 11L of DMSO for the 1000 reaction kit. This will produce a 4 mM substrate stock solution.
   b. Dilute the DMSO substrate stock with modified MAO-B Reaction Buffer to a concentration of 16 11M (25Qx dilution). For example, to perform a 96-well plate of reactions for MAO-B, use 5.0 11L of MAO substrate stock diluted to a final volume of 1250 IlL.
3. **Luciferin Detection Reagent:**
   a. Equilibrate the Reconstitution Buffer with Esterase and lyophilized Luciferin Detection Reagent to room temperature.
   b. Transfer the entire contents of the bottle of Reconstitution Buffer with Esterase to the amber bottle of lyophilized Luciferin Detection Reagent. Mix by swirling or inverting several times to obtain a homogeneous solution. Do not vortex or shake.

4. 4x **Selegiline (SEL) Stock Solutions:** Selegiline is used as positive control inhibitor of MAO-B
   a. 40 mM selegiline (SEL) stock (400x):- Dissolve 17.9 rng SEL HCl (MW 223.7 g/mol) in 2.0 rnL ofDMSO.
   b. –Prepare 4x SEL solutions in MAO-B Reaction Buffer.

Final cone. (uM)	4x Stock (uM)	uL of stock	uL MAO-B Buffer
100	400	10 L of 40 rnM	990 L
10	40	50 L of 400 J..LM	450 J..LL
▮	4	50 J..LL of 40 J..LM	450 L
0.1	0.4	50 L of4 M	450 J..LL
0.01	0.04	50 L of 0.4 M	450 L
0 (vehicle control)	0	0	500 J..LL

5. 4x **Test Drug Stock Solutions:** It is recommended that test drug solubility in DMSO and assay buffer be tested before preparing incubation mixes. The top concentrations of drug may need to be adjusted or omitted if solubility is limited.
   a. Prepare a 400x stock of each test drug in DMSO.
   b. Prepare 4x stocks (as above for SEL) to give fmal assay concentrations of 0, 0.01, 0.10, 1.0, 10, and 100 M.

6. Thaw 2x **mouse brain homogenates** on ice; mix gently.

Incubation Procedures

1. Prepare incubation mixes:
   a. Test article incubations: Pipette the following into wells:
      - 12.5 J..LL of4x (16 M) substrate solution
      - 12.5 L of 4x test article solutions
   b. Positive SEL controls: Pipette the following into wells:
      - 12.5 L of 4x (16 M) substrate solution
      - 12.5 L of 4x SEL solutions
   c. No enzyme controls:
      - 12.5 L of 4x (16 J..LM) substrate solution
      - 12.5 L ofMAO-B Reaction Buffer
2. Incubate samples: Start reactions by adding 25 L of 2x mouse brain homogenate or 100 mM HEPES buffer, pH 7.5 (for no enzyme controls) to each well; mix. Incubate 120 minutes with shaking at ambient temperature.
3. Process and analyze samples:

a. Add 50 11L of reconstituted Luciferin Detection Reagent to each well of the plate and mix briefly.

b. Incubate the plate at room temperature for 20 minutes and measure the luminescence of the wells.

4. Calc ulations:

a. Using raw relative luminescence unit (RLU) values, determine means and standard deviations for no enzyme controls.

b. Correct vehicle controls (0 11M) sample and positive control RLU values by subtracting the mean no enzyme control values.

c. Calculate percent of vehicle control values by dividing corrected sample and positive control RLU values by the mean RLU value of the corresponding vehicle control.

d. Determine the means and standard deviations of the percent of vehicle control values for test drug and positive control treatments.

References

1. Valley, M.P., Zhou, W., Hawkins, E. M., Shultz, J., Cali, J. J., Worzella, T., Bernad, L., Good, T., Good, D., Riss, T.L., Klaubert, D. H., and Wood, K. V. (2006). A bioluminescent assay for monoamine oxidase activity. *Ana/yt. Biochem.* **359,** 238-46.

2. Quinn, L. P., Crook, B., Hows, M. E., Vidgeon-Hart, M., Chapman, H., Upton, N., Medhurst, A. D., and Virley, D. J. (2008). The PPARy agonist pioglitazone is effecti ve in the MPTP mouse model of Parkinson's disease through inhibition of monoamine oxidase B. *Brit. J Pharmacol.* **154,** 226-233.

Appendix 1: Assay Validation Results

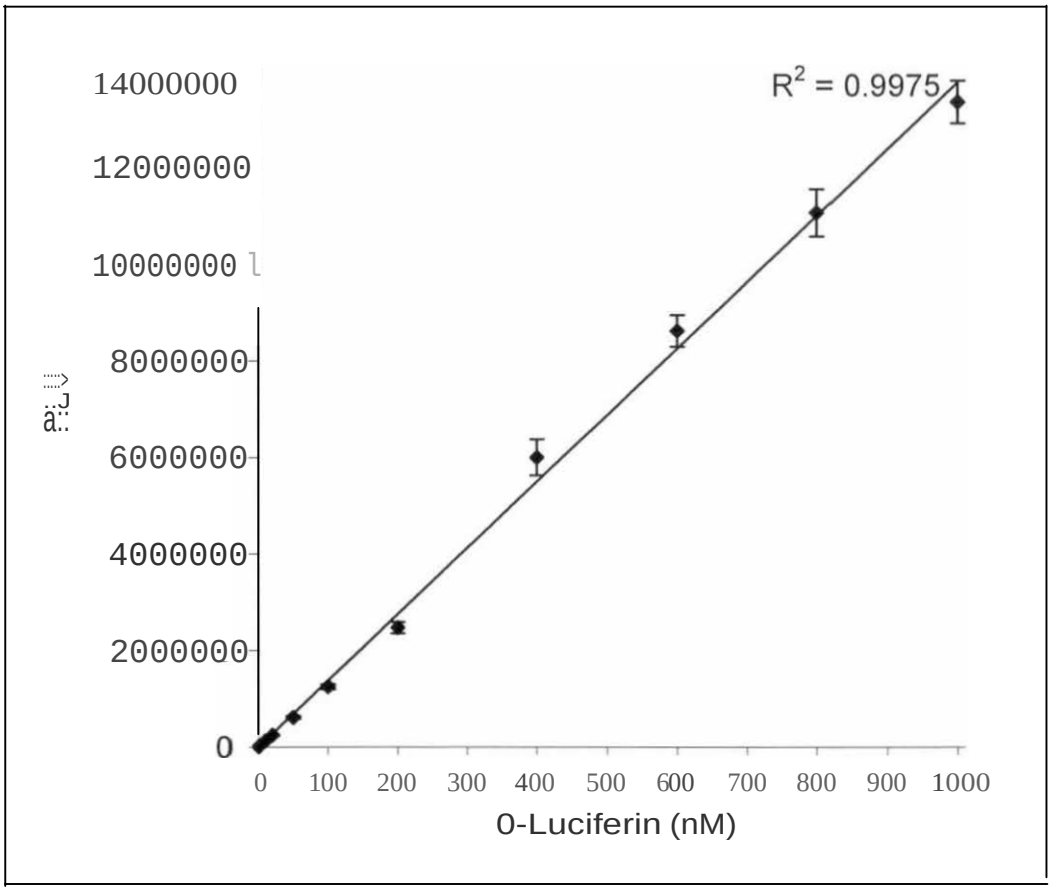

Figure 1: Linearity of luminescent response for a D-luciferin standard curve. The assay gives a linear response up to about 1.2×10^7 relative light units (RLU).

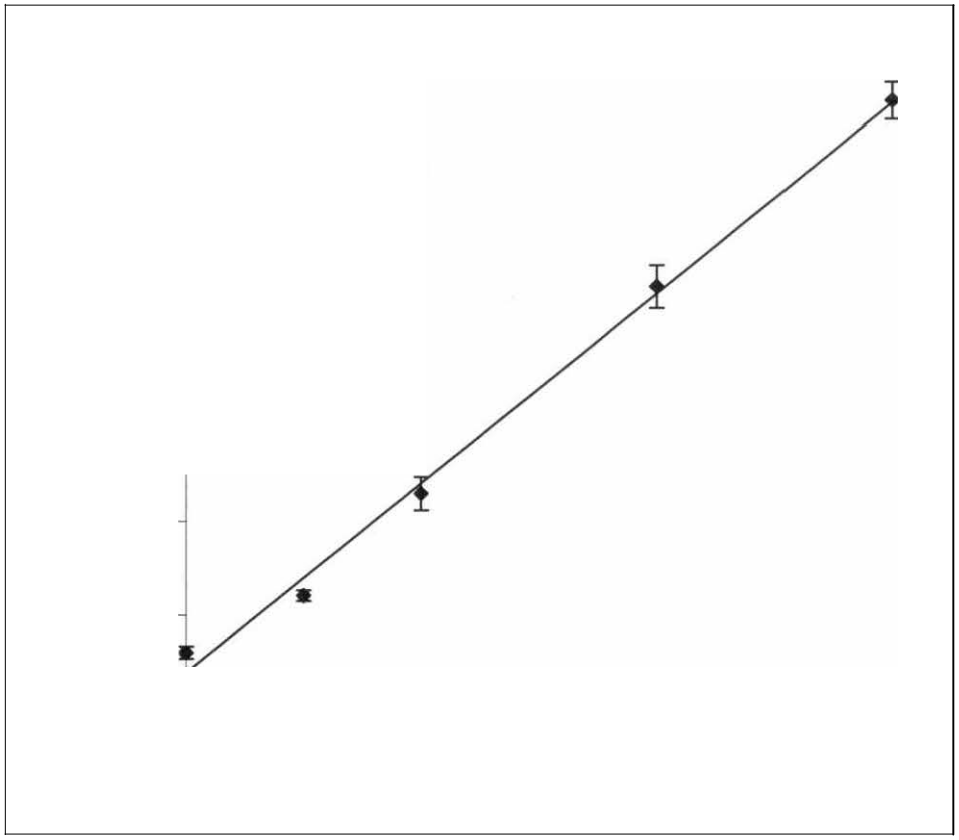

Figure 2: Time-dependence of assay response. The linear dependence of the reaction rate on time indicates that initial rate conditions have been satisfied for the assay, which is required for accurate determination of the Michaelis-Menten constant, Km. Based on these results, an incubation time of 2 hours was chosen.

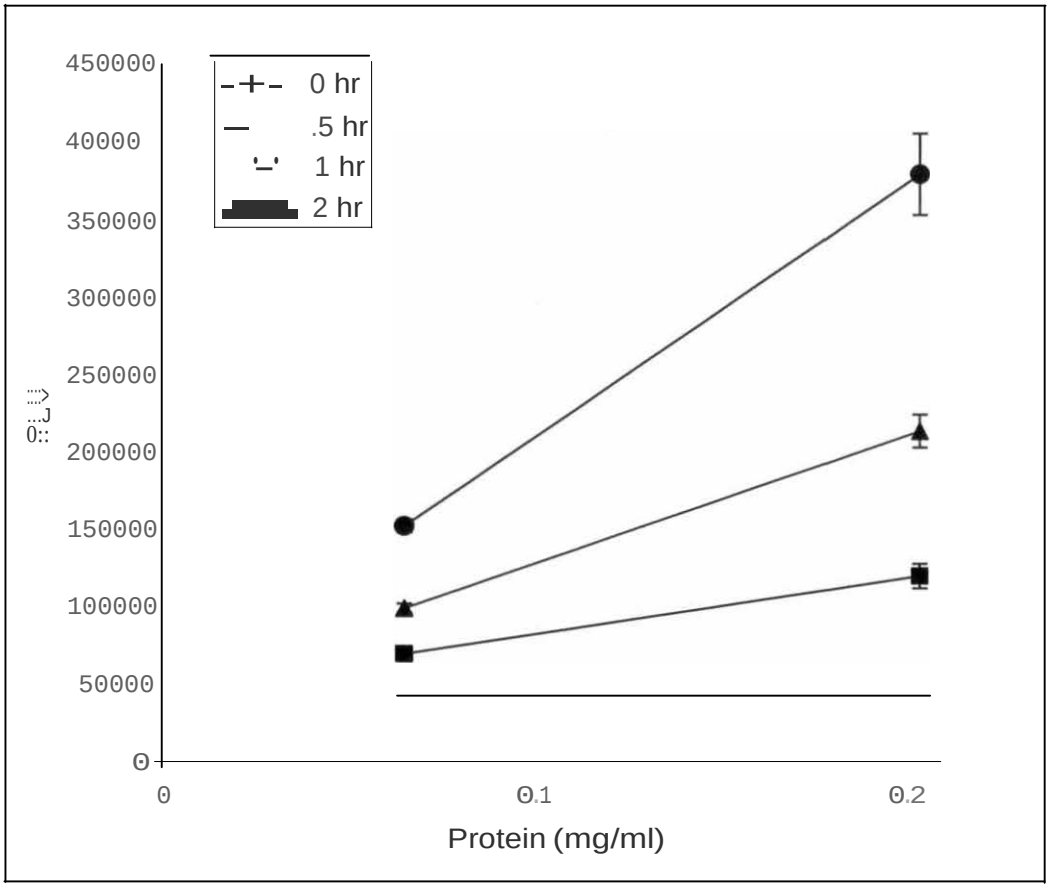

Figure 3: Protein-dependence of assay response. The dependence of the reaction rate on protein concentration also suggests that initial rate conditions have been satisfied for the assay. The range of linearity was limited by the lower response at lower protein concentrations and by interference of unknown origin at higher protein concentrations. Therefore, a fixed protein concentration of 0.2 mg/mL (10 f.lg protein/well) was chosen.

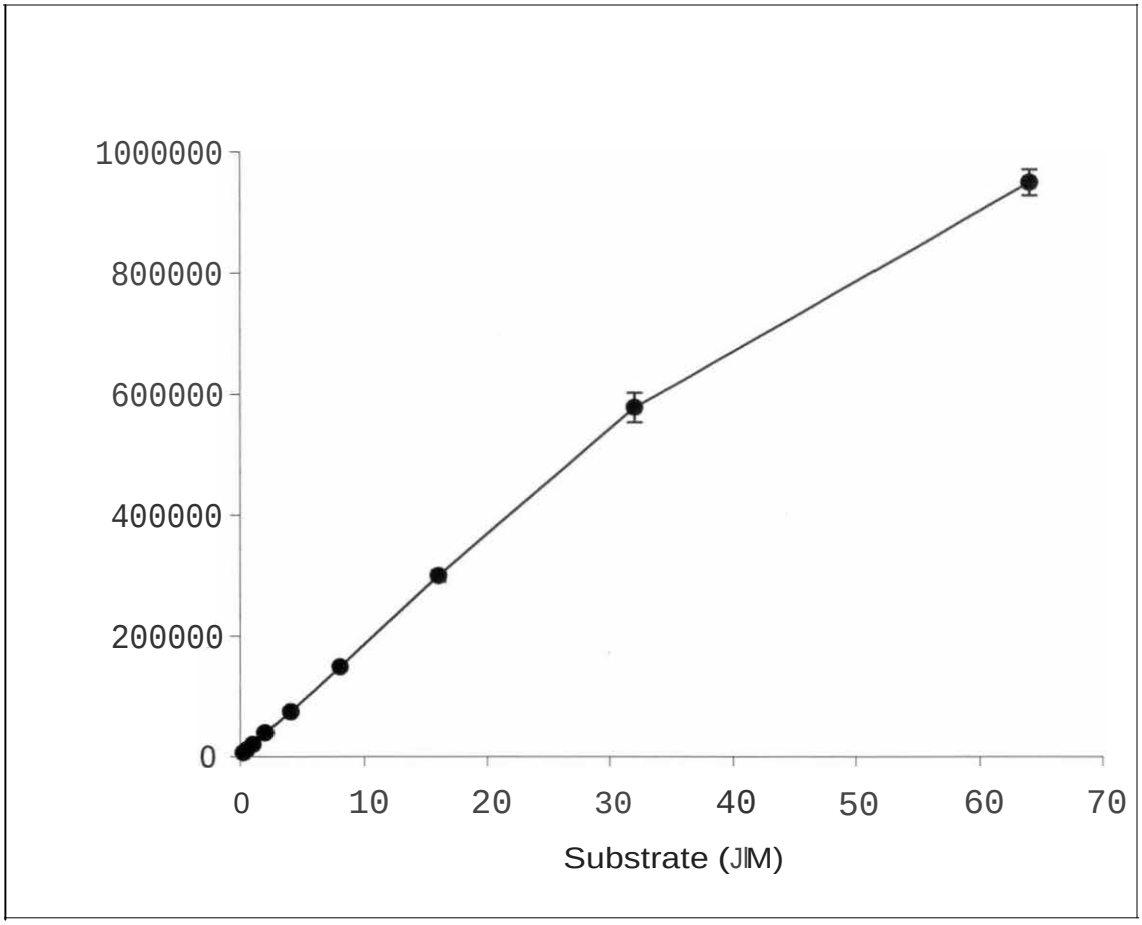

Figure 4: Substrate-dependent background response. The time- and protein-dependence experiments showed a background luminescence that was not dependent on time or protein concentration. The lwninescence was found to be dependent on the concentration of substrate in the absence of protein, and subsequent calculations corrected for this background.

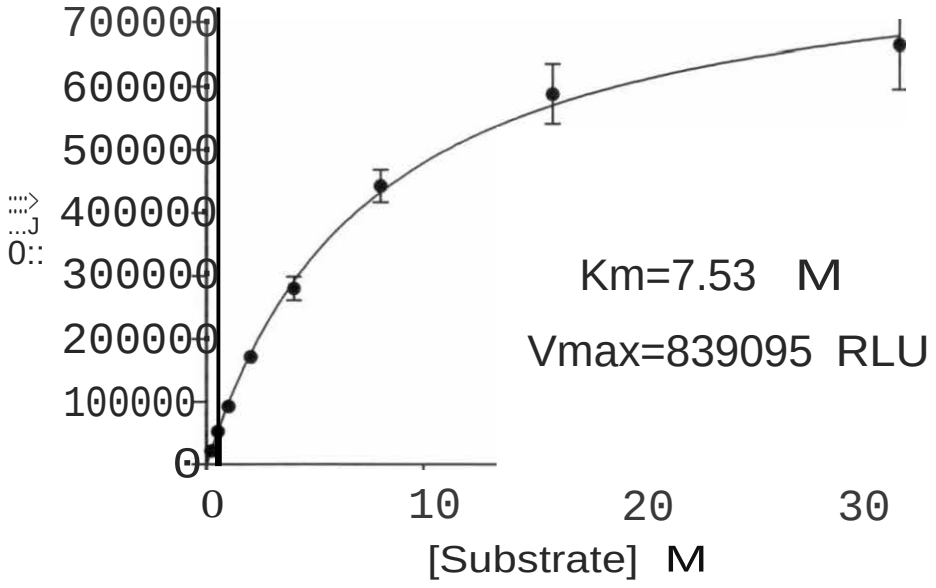

Figure 5: Substrate-velocity plot of Mouse Brain MAO-B assay. Under standard reaction conditions the Michaelis-Menten constant, Km, was determined to be 7.53 1-1M, and a substrate concentration of 4.0 1-1M was chosen for the mouse brain MAO-B inhibition assay.

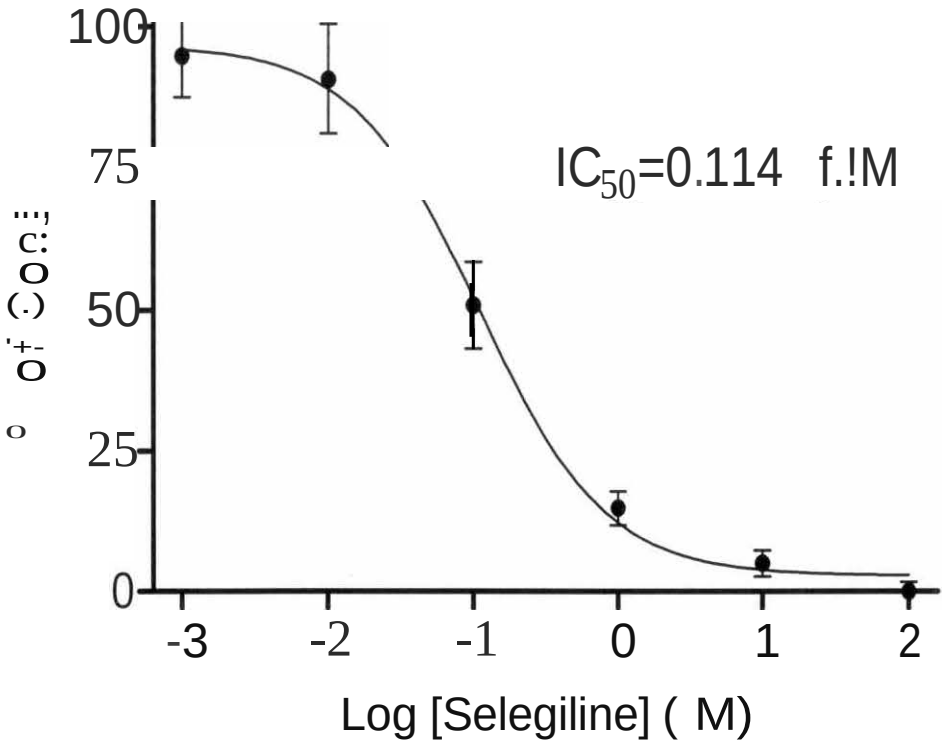

Figure 6: Selegiline inhibition of Mouse Brain MAO-B. Selegiline, a selective inhibitor of MAO-B, produced a concentration-dependent inhibition of mouse brain MAO-B with an IC_{50} value of0.114 f.!M.