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Analysis of Protein Expression in Brain Tissue by ELISA

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1. Introduction

The enzyme-linked immunosorbent assay (ELISA) technique offers a sensitive, simple, and versatile method for quantifying as little as 100 pg of target protein in mixed cell or tissue lysates. A further advantage to the protocol is the ability to process rapidly and reproducibly large numbers of samples with minimal equipment requirements. The underlying principle depends on formation of an antigen–antibody complex immobilized on plastic microtitre plates. **Figure 1** illustrates the basic methodology. In the antibody-sandwich ELISA, a primary antibody directed against a protein of interest is bound (adsorbed) to the bottom of a polystyrene well (**Fig. 1A**). A mixed protein lysate is added to the well and the target protein is "captured" onto the solid phase by interaction with the capture antibody (**Fig. 1B**). A second antibody recognizing a different antigenic determinant on the target protein is added (**Fig. 1C**). The resulting antibody–antigen–antibody sandwich is detected with an enzyme-linked secondary antibody (**Fig. 1D**) followed by incubation with a suitable enzyme substrate. Substrate hydrolysis results in a detectable color change (**Fig. 1E**) and is proportional to the amount of captured protein. At each step, the antibody sandwich is separated simply and effectively from unbound conjugate by repeated washes. Sensitivity can be increased further by secondary and tertiary immunogenic enhancement.

In this chapter, we describe standard protocols for protein detection in lysates prepared from microdissected brain tissue using antibody-sandwich ELISAs. To illustrate methods, cerebral changes in expression of connexin32 (Cx32) following chronic cocaine self-administration are presented. Connexins

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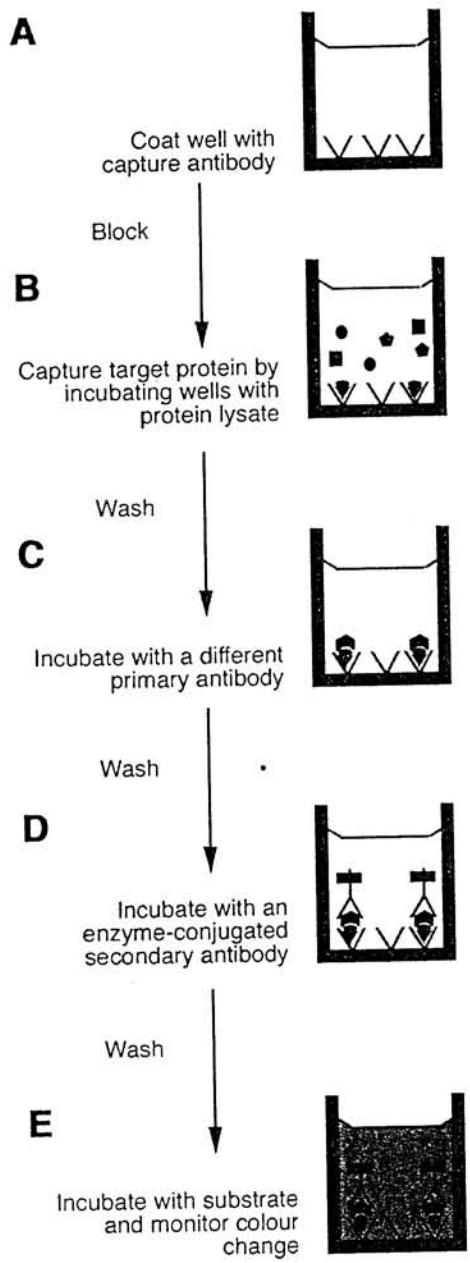

Fig. 1. Basic principles of the antibody-sandwich ELISA. The antibody-sandwich protocol is the most sensitive of the ELISA methods.

are a multigene family of more than 20 proteins that form the structural units of gap junction channels. These intercellular channels directly connect the cytoplasm of adjacent cells. Gap junctional intercellular communication (GJIC) allows coupled cells to synchronize their responses to extracellular cues by passive diffusion of ions, metabolites, and second messengers from one cell to another *(1)*. Administration of drugs of abuse has been shown to influence functional measures of GJIC between neurons in the striatum *(2,3)* although the individual connexin proteins responsible for these changes have only begun to be elucidated *(4)*. ELISA methodology represents one means of identifying these connexins.

2. Materials

1. Sodium pentobarbital (Somnotol, MTC Pharmaceuticals, Cambridge).
2. Dry ice.
3. Isopentane (2-methylbutane).
4. Razor blades.
5. Disposable borosilicate glass culture tubes (12 × 75 mm, VWR).
6. Tissue-Tearer hand-held small sample laboratory homogenizer (Fisher).
7. 10 m*M* Phosphate-buffered saline (PBS): 10 m*M* sodium phosphate; 150 m*M* NaCl, pH 7.4.
8. RIPA buffer: 10 m*M* PBS, 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium doedecyl sulfate (SDS). Buffer can be stored at 4°C. Add protease inhibitors and vortex-mix vigorously immediately before use. Protease inhibitors: Aprotinin (30 µL/mL from Sigma stock, stored at 4°C), 10 m*M* sodium orthovanadate (add 10 µL/mL of 100 m*M* stock to RIPA buffer, stored at –20°C), and phenylmethanesulfonyl fluoride (PMSF) (add 100 µL/mL of 10 mg/mL stock dissolved in isopropanol to RIPA buffer, stored at –20°C). **Warning**: PMSF is a neurotoxin. Take appropriate safety precautions when dissolving stock powder.
9. Bio-Rad DC protein assay kit (Bio-Rad, Mississauga, ON).
10. 96-Well microtitre plates.
11. Adhesive covers (available from any company supplying microtiter plates) or plastic wrap.
12. Capture primary antibody (rat monoclonal R5, kindly provided by Dr. David Paul, Harvard Medical School), detection primary antibody (mouse monoclonal M12.13, kindly provided by Dr. David Paul, Harvard Medical School), peroxidase-linked anti-mouse IgG secondary antibody (Jackson Immunolabs, Mississauga, ON).
13. 3,3′,5,5′-Tetramethylbenzidine (TMB). TMB can be purchased as a ready to use solution (BM Blue POD Substrate, Roche).
14. Coating buffer: 0.1 *M* $NaHCO_3$–Na_2CO_3, pH 9.5.
15. Blocking solution: 1% bovine serum albumin (BSA) (w/v) in 10 m*M* PBS, pH 7.4, and 0.02% thimerosol (w/v).

16. Multichannel pipet.
17. Plate reader with filters for detection at 450 nm and 650 nm.

3. Methods

Methodologies are described for extraction of protein and RNA from rodent striatum (**Subheading 3.1.**), protein capture onto microtiter plates (**Subheading 3.2.**), detection of bound antibody–antigen complex (**Subheading 3.3.**), and data analysis (**Subheading 3.4.**).

3.1. Protein Extraction from Rat Brain

This protocol includes a description of the euthanasia procedure (**Subheading 3.1.1.**), dissection parameters (**Subheading 3.1.2.**), and protein extraction methodology (**Subheading 3.1.3.**).

3.1.1. Animal Euthanasia

Male Wistar rats (275–300 g, Charles Rivers, St Foie, PQ) were lethally injected intraperitoneally with 80 mg/kg of sodium pentobarbital (Somnotol) and euthanized by decapitation. Brains were rapidly removed for dissection. In the present example, rats had been previously allowed to self-administer 38 injections of 1.5 mg/kg of cocaine over a 14-d period *(4)*. Animals were killed at 2, 7, or 21 d after the last cocaine self-administration session and Cx32 protein expression was compared to drug-naive, age-matched controls *(4)*.

3.1.2. Tissue Dissection

The medulla, parietal/temporal cortex, cerebellum, thalamus, dorsal striatum, nucleus accumbens, and globus pallidus/ventral lateral striatum (not including the nucleus accumbens) were removed under a dissecting microscope, weighed, flash-frozen in dry-ice chilled isopentane, and stored at –85°C until processing.

3.1.3. Protein Extraction

Lysates of soluble protein were prepared from pooled tissue samples (n = 2 animals/protein lysate). The steps in this process involve homogenization, separation of unhomogenized tissue and insoluble proteins from soluble protein lysate, and determination of protein concentration. The following protocol gives the highest protein yield from small samples with minimal extraction steps (*see* **Note 1**).

1. Place tissue on a clean glass plate chilled on ice.
2. Dice tissue into small pieces with a razor blade. Wipe plate with 100% ethanol (EtOH) between samples.
3. Immediately transfer diced tissue to disposable borosilicate glass culture tubes (12 × 75 mm) (VWR) and add RIPA buffer with protease inhibitors (3 mL/g of tissue). As it is difficult to homogenize tissue in <500 µL of RIPA (~170 mg of tissue), it may be necessary to pool multiple dissections from different animals to

achieve ~170 mg of tissue. Samples should be maintained on ice for the duration of the extraction procedure.

4. Homogenize tissue using a hand-held or stand-mounted homogenizer. The settings and number of strokes must be empirically determined depending on the unit employed. Using a Tissue-Tearer hand-held small sample laboratory homogenizer (Fisher) with a probe length of 8.3 × 0.7 cm, 8–10 up-and-down motions (strokes) through the tissue solution at setting 3 is sufficient to homogenize small samples of myelinated tissue (~170 mg tissue/500 µL of RIPA buffer). Large samples such as cortex or cerebellum may require additional homogenization. Care should be taken to ensure that the probe is not removed from the liquid or excess frothing will occur. Samples are subjected to three to five strokes and solid tissue and froth bubbles are allowed to settle on ice prior to renewed homogenization. Carefully clean pestle with 70% EtOH and double-distilled H_2O (2X H_2O) between samples to eliminate cross-contamination.
5. Transfer homogenized lysates to 1.5-mL microcentrifuge tubes and centrifuge at 12,000 rpm for 20 min at 4°C. Remove supernatant and microfuge again. The second supernatant is the total cell lysate used in the ELISA protocol.
6. Combine lysates from the same sample. Determine protein concentration using standard laboratory protocol such as the Bio-Rad DC protein assay kit (Mississauga, ON). If homogenization has been performed properly, the final protein concentration should be between 0.5 and 1 µg/µL. A yield of ≥50 µg of total protein/200 mg of starting tissue is commonly achieved.
7. Aliquot samples for storage. Aliquots can be frozen at –20°C for up to 3 mo without significant degradation of connexin proteins. Repeated freeze–thaw should be avoided.

3.2. Protein Capture onto Microtiter Plates

In the protocol described, Cx32 protein was immobilized on 96-well polystyrene plates coated with rat monoclonal R5 (provided by Dr. David Paul, Harvard Medical School). This assay was designed not to quantify absolute protein levels but to establish whether relative Cx32 expression changes over time in various tissues following cocaine self-administration *(4)*. As a result, a standard curve was not included. If a sufficient amount of purified protein is available, this protocol can be modified to quantify absolute protein concentration by inclusion of a standard curve with known amounts of target protein (*see* **Subheading 3.2.2., step 1**).

3.2.1. Adsorb the Capture Antibody onto the Solid Phase of a Microtiter Plate

1. Dilute capture primary antibody to 2.5 µg/mL in coating solution (*see* **Note 2**).
2. Add 50 µL per well using a multichannel pipet (12-channel 20–200-µL Costar Pipettor). Agitate plate to cover the surface of the well evenly with coating solution.

3. Cover the plate with plastic adhesive covers or plastic wrap and allow antibody to bind to the plastic by incubating at 4°C overnight.
4. Remove the coating solution by turning the plate upside down over a sink and shaking. Strike the plate against a paper towel three times to remove as much of the remaining coating solution as possible. A sharp, definitive striking motion is optimal.
5. Wash plates with 10 m*M* PBS using a multichannel pipet. Add 100 µL per well. Remove wash solution as described in **step 4**. Repeat for a total of three washes.
6. Block the residual binding capability of the wells with 100 µL/well in 1% BSA in 10 m*M* PBS containing 0.02% thimerosol as a preservative. Cover the plate with plastic adhesive covers or plastic wrap and incubate at 37°C for 1 h. Remove the plate to room temperature if more time is required to prepare subsequent reagents. Plates can also be prepared in advance and left in blocking solution overnight at 4°C.

3.2.2. Immobilize Target Protein

1. Prepare 20 µg of protein lysate per well. Adjust all samples to the same volume of RIPA buffer before addition of coating buffer. Bring each sample to a final volume of 50 µL with coating buffer. If a standard curve is required, it should be prepared at this step (*see* **Note 3**).
2. Remove the blocking solution as described in **step 4**.
3. Add 50 µL of test samples to antibody-coated wells in duplicate or triplicate to control pipetting errors. A sample plate layout is provided in **Table 1**. Include a standard curve on each plate if required (*see* **Note 3**). Cover the plate with plastic adhesive covers or plastic wrap and incubate ≥ 2 h at room temperature or overnight at 4°C. Include multiple wells incubated with coating buffer only as blanks (**Table 1**).

3.3. Detection of Bound Antigen–Antibody Complex

Detection of bound antibody–antigen complex can be direct or indirect. In both cases, the captured antibody–protein complex is incubated with a primary detection antibody capable of recognizing different antigenic determinants on the target protein than the capture antibody. The epitopes must remain available after the target protein is immobilized on the microtiter plate and, as a result, not all antibody pairs are compatible (*see* **Note 2**).

Direct detection is accomplished using a primary detection antibody that is already conjugated to peroxidase or alkaline phosphatase. Advantages are a faster and easier assay with fewer steps as well as the freedom to use capture and detection antibodies raised in the same species. Disadvantages include reduced sensitivity relative to the indirect method and practical concerns in that an appropriate conjugated antibody may not be available.

Table 1
Microplate Layout for Experimental Detection of Cx32 in Medulla, Parietal/Temporal Cortex, Cerebellum, Thalamus, Dorsal Striatum, Nucleus Accumbens, and Globus Pallidus/Ventral Lateral Striatum in Drug-Naive (Control) Animals and in Chronic Cocaine Self-Administrators After 2, 7, and 21 d of Cocaine Withdrawal

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0 µg protein	M Control 20 µg	M Control 20 µg	CB Control 20 µg	CB Control 20 µg	DS Control 20 µg	DS Control 20 µg	VS Control 20 µg	VS Control 20 µg		
B	Blank	0 µg protein	M 2 d 20 µg	M 2 d 20 µg	CB 2 d 20 µg	CB 2 d 20 µg	DS 2 d 20 µg	DS 2 d 20 µg	VS 2 d 20 µg	VS 2 d 20 µg		
C	Blank	0 µg protein	M 7 d 20 µg	M 7 d 20 µg	CB 7 d 20 µg	CB 7 d 20 µg	DS 7 d 20 µg	DS 7 d 20 µg	VS 7 d 20 µg	VS 7 d 20 µg		
D	Blank	0 µg protein	M 21 d 20 µg	M 21 d 20 µg	CB 21 d 20 µg	CB 21 d 20 µg	DS 21 d 20 µg	DS 21 d 20 µg	VS 21 d 20 µg	VS 21 d 20 µg		
E	Blank	0 µg protein	Cx Control 20 µg	Cx Control 20 µg	T Control 20 µg	T Control 20 µg	NA Control 20 µg	NA Control 20 µg				
F	Blank	0 µg protein	Cx 2 d 20 µg	Cx 2 d 20 µg	T 2 d 20 µg	T 2 d 20 µg	NA 2 d 20 µg	NA 2 d 20 µg				
G	Blank	0 µg protein	Cx 7 d 20 µg	Cx 7 d 20 µg	T 7 d 20 µg	T 7 d 20 µg	NA 7 d 20 µg	NA 7 d 20 µg				
H	Blank	0 µg protein	Cx 21 d 20 µg	Cx 21 d 20 µg	T 21 d 20 µg	T 21 d 20 µg	NA 21 d 20 µg	NA 21 d 20 µg				

Layout of a 96-well plate and loading of experimental samples as described in **Subheading 3.2.2.** is illustrated. Blank: Wells were coated with R5 capture antibody and blocked with blocking solution. In all subsequent steps, wells were incubated with the appropriate buffers in the absence of protein lysate or antibodies. These values are set to 0 by the microplate reader during the run. 0 µg protein: Wells were treated exactly as described for experimental samples except protein was not included in the 40 µL of RIPA and 10 µL of coating buffer (**Subheading 3.2.2., step 1**). This control determines the lower limit of assay sensitivity and establishes nonspecific binding of all reagents in the absence of target protein. Experimental values that fall below this value cannot be interpreted. If this absorbance is high, increase the number of washes. M, Medulla; Cx, parietal–temporal cortex; CB, cerebellum; T, thalamus; DS, dorsal striatum; NA, nucleus accumbens; VS, ventral striatum (not including the nucleus accumbens). Control: Samples obtained for drug-naive animals. 2 d: Protein extraction performed 2 d after the last cocaine injection. 7 d: Protein extraction performed 7 d after the last cocaine injection. 21 d: Protein extraction performed 21 d after the last cocaine injection. Experimental samples represent protein lysates extracted from tissue pooled from two animals.

Indirect detection is accomplished using an unconjugated detection antibody raised in a different species to the capture antibody followed by incubation with an enzyme-conjugated secondary antibody that recognizes the detection antibody. Sensitivity can be enhanced further by tertiary reaction (*see* **Note 4**). The advantage to this method lies in its sensitivity. Disadvantages include increased assay processing time and labor as well as the requirement to identify capture and detection antibodies raised in different species.

The protocol provided below is an example of secondary enhancement. Cx32 protein, immobilized on 96-well polystyrene plates coated with monoclonal R5 raised in rat (*see* **Subheading 3.2.**), was reacted with mouse monoclonal M12.13. Antibody–antigen–antibody complexes were detected with a peroxidase-linked anti-mouse IgG (1:2000) and TMB.

1. Remove unbound lysate by turning the plate upside down over a sink and shaking. Strike the plate against a paper towel several times to remove as much of the remaining lysate as possible.
2. Wash plates with 10 m*M* PBS using a multichannel pipet. Add 100 µL per well. Remove wash solution as described in **step 4**. Repeat for a total of three washes.
3. Dilute detection primary antibody to 2.5 µg/mL in blocking solution (*see* **Note 2**).
4. Add 50 µL per well using a multichannel pipet (12-channel 20–200-µL Costar Pipettor). Agitate plate to cover the surface of the well evenly with solution. Cover the plate with plastic adhesive or plastic wrap and incubate ≥2 h at room temperature.
5. Remove unbound antibody and wash plates as described in **steps 1** and **2**.
6. Detect the antibody–antigen–antibody complexes with peroxidase-linked anti-mouse IgG (1:2000) or appropriate secondary antibody raised against the Ig (i.e., Ig, IgG, IgM, etc.) and species (i.e., mouse, rabbit, sheep, etc.) of the detection primary antibody. Add 50 µL per well. Cover with plastic adhesive or wrap and incubate for 2 h at 37°C.
7. Remove unbound antibody and wash plates as described in **steps 1** and **2**.
8. Add 75 µL per well of TMB substrate and incubate for 10–30 min at room temperature.
9. Stop reaction by addition of 25 µL of 1 *M* H_2SO_4. The solution will turn a yellow color.
10. Read the plate on a microplate reader at 450 nm against a reference wavelength of 650 nm.

3.4. Data Analysis

In the example provided, -fold changes in Cx32 expression were assessed in medulla, parietal/temporal cortex, cerebellum, thalamus, dorsal striatum, nucleus accumbens, and globus pallidus/ventral lateral striatum (not including

Table 2
Raw Data from a Representative Antibody-Sandwich ELISA Experiment

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	0 µg protein	M Control	M Control	CB Control	CB Control	DS Control	DS Control	VS Control	VS Control		
	0.000	0.027	0.850	0.851	0.641	0.834	0.651	0.525	0.602	0.891		
B	Blank	0 µg protein	M 2 d	M 2 d	CB 2 d	CB 2 d	DS 2 d	DS 2 d	VS 2 d	VS 2 d		
	0.000	0.022	0.947	0.913	1.009	0.983	0.577	0.576	0.972	1.016		
C	Blank	0 µg protein	M 7 d	M 7 d	CB 7 d	CB 7 d	DS 7 d	DS 7 d	VS 7 d	VS 7 d		
	0.001	0.019	0.938	0.913	0.855	0.675	0.644	0.615	0.972	0.986		
D	Blank	0 µg protein	M 21 d	M 21 d	CB 21 d	CB 21 d	DS 21 d	DS 21 d	VS 21 d	VS 21 d		
	−0.003	0.022	0.971	0.774	0.785	0.582	0.560	0.704	0.923	0.834		
E	Blank	0 µg protein	Cx Control	Cx Control	T Control	T Control	NA Control	NA Control				
	−0.001	0.019	0.629	0.938	0.572	0.813	0.976	0.974				
F	Blank	0 µg protein	Cx 2 d	Cx 2 d	T 2 d	T 2 d	NA 2 d	NA 2 d				
	0.002	0.078	0.829	0.821	0.897	0.952	0.938	0.959				
G	Blank	0 µg protein	Cx 7 d	Cx 7 d	T 7 d	T 7 d	NA 7 d	NA 7 d				
	0.000	0.022	0.815	0.715	0.960	1.011	0.916	0.913				
H	Blank	0 µg protein	Cx 21 d	Cx 21 d	T 21 d	T 21 d	NA 21 d	NA 21 d				
	−0.001	0.021	0.954	0.677	0.998	0.843	0.843	0.824				

Layout and abbreviations are as described in **Table 1**. Data represent well absorbance read at 450 nm minus absorbance read at a reference wavelength of 650 nm. For subsequent data analysis, the mean of replicate wells defines a single data point.

nucleus accumbens) at various time points after cocaine withdrawal. **Table 2** depicts raw data from a single experiment. Examples of data analysis and interpretation are presented in **Figs. 2** and **3**. Graphs depict averaged data from two independent experiments (two antibody-sandwich ELISAs) using different protein lysates. In **Fig. 2A–C**, mean absorbances and data standardized to Cx32 levels in drug-naive control animals are presented. To perform standardization calculations, each data point/experiment/tissue was divided by the mean of the control group (drug-naive animals). This transformation sets control expression levels to 1.0 and permits calculation of both average -fold change relative to control and standard error of the mean for each condition. Standardization simplifies comparisons of changes in protein expression between tissues that express different amounts of protein under basal conditions (compare **Fig. 2A** with **2B**). If a standard curve is included in the experimental design, -fold

A
ABS
1
0.8
0.6
0.4
0.2
0
Dorsal Striatum
Nucleus Accumbens
Globus pallidus/ventral lateral striatum
B
Dorsal Striatum
Nucleus Accumbens
Globus pallidus /ventral lateral striatum
ABS
1.000
0.800
0.600
0.400
0.200
0.000
0
7
14
21
Days after last cocaine injection
C
Dorsal Striatum
Nucleus Accumbens
Globus pallidus /ventral lateral striatum
Fold change in Cx32 protein expression
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0
7
14
21
Days after last cocaine injection

change can be replaced with precise quantitation of the protein concentration in each sample (*see* **Note 2**).

4. Notes

1. Alternative protocols for protein extraction include the use of Trizol Reagent (Invitrogen, Burlington, ON) permitting simultaneous extraction of RNA and protein from the same tissue sample and minimizing the number of animals required to perform both RNA and protein studies *(4)*. However, because Trizol-extracted protein is solubilized in 1% SDS, care should be taken to ensure that the final concentration of SDS does not exceed 0.5% when samples are diluted in coating buffer (**Subheading 3.3.2., step 1**). Stripping of capture antibody from the microtiter plates and loss of antibody–antigen complex can occur at elevated SDS concentrations.
2. The capture antibody can be either monoclonal or polyclonal and is diluted to a concentration between 0.5 and 10 µg/mL. As a rule, we have observed that 2.5 µg/mL is a convenient starting concentration and that sensitivity does not significantly increase when concentrations exceed 10 µg/mL. If the capture and detection antibodies have not previously been used for ELISA analysis, it may be necessary to establish the limits of sensitivity using serial dilutions of both reagents detecting a known positive control. Note that the sensitivity of the

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Fig. 2. *(previous page)* Analysis and interpretation of antibody-sandwich ELISA results. **(A)** Distribution of Cx32 protein in the dorsal striatum, nucleus accumbens, and globus pallidus/ventral lateral striatum (minus nucleus accumbens) in drug-naive animals. Data represent the mean absorbance ± SEM of two independent experiments. Note that the expression levels of Cx32 vary under control conditions. The limit of sensitivity (i.e., the average of wells labeled 0 µg of protein in **Table 2**) is illustrated by the line at the bottom of the graph. Absorbances below this limit can not be distinguished from nonspecific binding (*see* Table 1). **(B)** Alterations in Cx32 expression following withdrawal from chronic cocaine self-administration. Data are expressed as the mean absorbance ± SEM of two independent experiments. The limit of sensitivity is indicated by the horizontal line at bottom of the graph. As presented, it is difficult to compared changes in Cx32 expression following cocaine withdrawal. **(C)** -Fold changes in Cx32 expression in the dorsal striatum, nucleus accumbens, and globus pallidus/ventral lateral striatum during cocaine withdrawal. Standardization permits a clearer comparison of trends in Cx32 expression at various time points after cocaine self-administration. Note that relative protein expression does not change in the dorsal striatum during cocaine withdrawal but that expression decreases over time to below that observed in drug-naive animals in the nucleus accumbens. These data replicate previously published results *(4)*. In the globus pallidus/ventral lateral striatum, a sharp increase in Cx32 expression is observed immediately after cocaine withdrawal followed by a gradual decrease to basal protein levels.

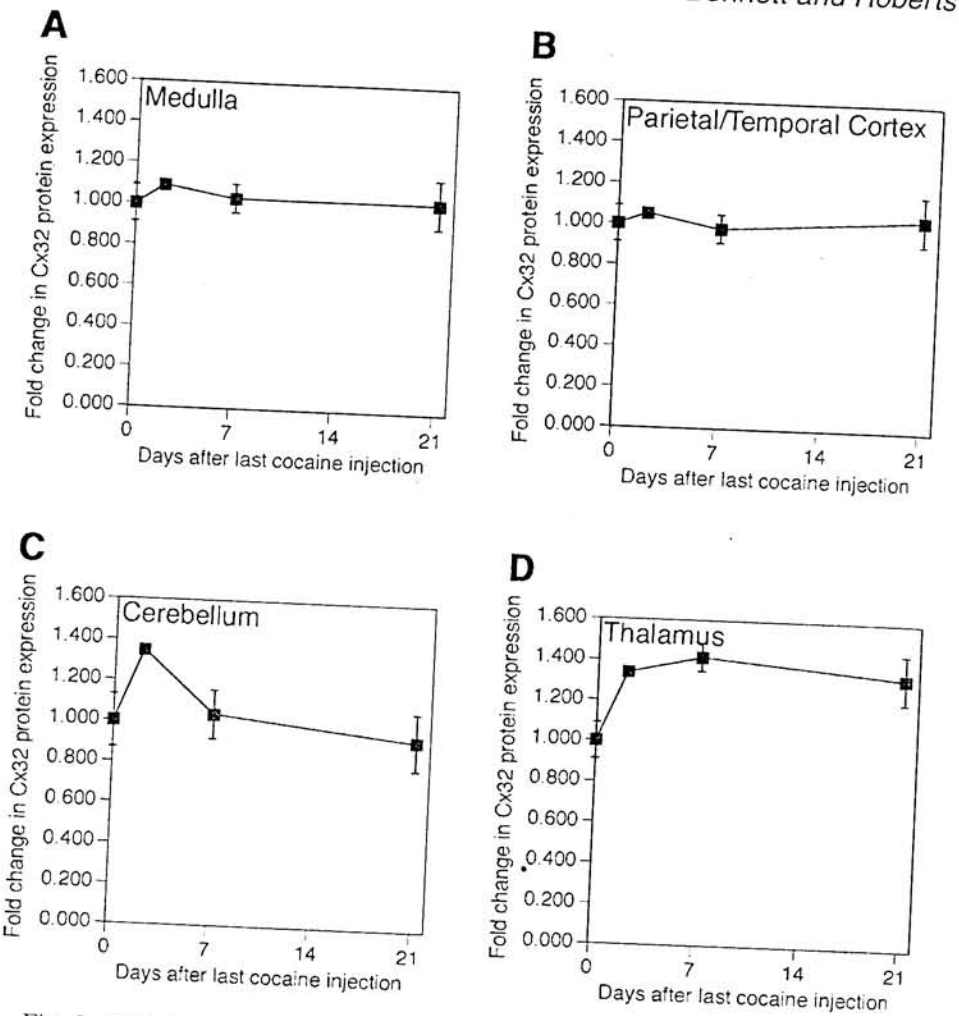

Fig. 3. Fold changes in Cx32 expression in the medulla, parietal/temporal cortex, cerebellum, and thalamus. (**A**) Medulla. (**B**) Parietal/temporal cortex. (**C**) Cerebellum. (**D**) Thalamus. Note that there is no change in Cx32 expression in the medulla or parietal/temporal cortex relative to drug-naive animals during cocaine withdrawal. A transient increase in expression is observed 2 d after cocaine withdrawal in the cerebellum while sustained increases in Cx32 expression are noted in thalamic nuclei 2–21 d after cocaine withdrawal.

ELISA protocol depends on use of high-affinity antibodies with minimal cross-reactivity to other proteins and reagents and it is worth the time to optimize these reagents.

3. If the absolute amounts of protein are to be quantified, then a standard curve with serial dilutions of known amounts of target protein is required. However, binding

efficiency does vary from plate to plate and we have found that (a) a standard curve is required on each microtiter plate for accuracy and (b) it is essential that all of the experimental conditions are kept constant (i.e., incubation times). The standard curve establishes the dynamic range of detection (i.e., the amount of target protein required to produce linear differences in substrate hydrolysis) and permits quantitative assessment of target protein concentration in experimental lysates. The concentration of target protein in mixed protein lysates must fall within this dynamic range for accurate quantitation.

4. In the most sensitive of assays, detection is achieved by incubation with an unconjugated detection antibody, a biotinylated secondary antibody, and tertiary reaction with enzyme-conjugated streptavidin. This method enhances detection of a single antigen–antibody interaction up to eightfold. If tertiary enhancement is required, the modifications to the protocol outlined in **Subheading 3.3.** should be made:

   1–5. These steps remain unchanged.

   6. Amplify the antibody–antigen–antibody complex signal with biotinylated anti-mouse IgG (1:200,000, Sigma) or appropriate secondary antibody. Add 50 µL per well. Cover with plastic adhesive or wrap and incubate for 1 h at 37°C.
   7. Remove unbound antibody and wash plates as described in **steps 1** and **2**.
   8. Detect this complex with extravidin peroxidase (2 µg/mL, Sigma). Add 50 µL per well. Cover with plastic adhesive or wrap and incubate for 1 h at 37°C.
   9. Remove unbound antibody and wash plates as described in **steps 1** and **2**.
   10. Add 75 µL per well of TMB substrate and incubate for 10–30 min at room temperature.
   11. Stop reaction by addition of 25 µL of 1 *M* H_2SO_4. The solution will turn a yellow color.
   12. Read the plate on a microplate reader at 450 nm against a reference wavelength of 650 nm.

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