



A quantitative ELISA for bioactive anti-Nogo-A, a promising regenerative molecule for spinal cord injury repair

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ABSTRACT

The detection and quantification of bioactive anti-Nogo-A mAbs, which is of interest for the treatment of spinal cord injury, has previously been accomplished using cellular or indirect immunoassays. In one such assay the presence of Nogo-A inhibits neurite outgrowth from the PC12 neuronal cell line: pre-treatment with anti-Nogo-A overcomes this inhibition and the concentration of anti-Nogo-A is correlated with the reduction in growth inhibition. In the current work we demonstrate the first anti-Nogo-A sandwich ELISA utilizing a Nogo-A fragment in the role of capture agent and the anti-Nogo-A mAb 11c7 as the soluble analyte. Because the Nogo-A fragment contains the amino acid sequence against which 11c7 was raised, we postulate this combination reproduces the native binding mechanism and results in the detection of bioactive anti-Nogo-A. In support of this hypothesis, we have found good agreement between the inhibitory action of the Nogo-A fragment and myelin proteins used in existing PC12 cell assays. Importantly, unlike the several days required for cellular assays the ELISA is a fast and easy to use method for the detection and quantification of bioactive 11c7 in the range of 500–6000 pg/mL.

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1. Introduction

Traumatic spinal cord injury (SCI) affects 10,000 new patients annually in North America, often with devastating emotional and financial impact on the patient and family [1]. Although there is yet no cure for SCI, promising advances in tissue engineering and an enhanced understanding of the cellular mechanisms after injury have stimulated translation of basic discoveries into clinical trials [2,3].

Since the 1980s it has been known that central nervous system (CNS) neurons can extend neurites after injury when provided with a permissive environment such as that in a peripheral nerve graft [4]. The endogenous inhibition of the CNS has been recognized by the interaction of cell surface receptors on CNS neurons with myelin [5,6]. The transmembrane protein Nogo-A was identified as a primary contributor to neurite inhibition, and application of antibodies raised against this mole-

cule resulted in significant axonal growth *in vitro* and in animal models of SCI in both rat [7] and macaque monkey [8]. These and other studies demonstrated that anti-Nogo-A partially functions by blocking the interaction of Nogo-A with the Nogo receptor (NgR) and co-receptors p75 [9,10], TROY [11,12] and LINGO-1 [13] located on CNS neurons. Delivery of anti-Nogo-A results in increased neuroregeneration and thus represents an important regenerative strategy after SCI. Significantly, at least one mAb is being evaluated clinically in Europe where it is being delivered by external pump and catheter [14]. While our long term goals include developing a localized intrathecal delivery strategy [15,16], the focus herein is on a new method related to facilitate quantification of bioactive anti-Nogo-A by ELISA.

Various anti-Nogo-A antibodies have been reported in the literature including IN-1 [17] and 11c7 [18], among others. The mAb 11c7 was raised in mice against a synthetic peptide corresponding to the rat aa (amino acids) 623–640 of Nogo-A [18], which are within the so-called central inhibitory domain [14]. The *in vitro* presence of 11c7 has been determined by Western blot [19] or an indirect immunoassay utilizing brain sections [20], but more commonly by one of a variety of cell based assays utilizing PC12 neurons or 3T3 fibroblasts [18,19,21]. While cellular assays are valued because they quantify bioactive anti-Nogo-A, they are time intensive. The development of a fast and

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easy to use quantitative assay for bioactive 11c7 is therefore of interest to those working in SCI research for the determination of circulating antibody concentration *in vivo* and the development of drug delivery systems *in vitro*.

Enzyme linked immunosorbent assays (ELISA) have been widely used to quantify antigen concentrations in the range of picograms per milliliter [22]. In the current application, sandwich (or capture) ELISA is of special interest because the analyte is captured from solution by specific antigen/antibody binding, not by adsorption to a substrate. This feature allows the native binding of 11c7 to Nogo-A to be reproduced.

Rat Nogo-A is 1163 aa long with two regions known to inhibit neurite outgrowth. At least one of these, corresponding to aa 544–725 [18,23] is commercially available as a recombinant rat Nogo-A/Fc chimera: this inhibitory sequence is fused to the Fc region of human IgG [24]. We have used this Nogo-A chimera as the capture agent in a sandwich ELISA because it contains the 18 aa sequence used to generate 11c7 [18]. Thus in this newly developed ELISA, Nogo-A acts as the substrate-bound antigen and 11c7 as the soluble antibody analyte.

Given that binding of anti-Nogo-A to Nogo-A ameliorates abortive sprouting and allows neurons to extend neurites over Nogo-A containing substrates [7,8], the bioactivity of 11c7 is thus defined by its ability to bind Nogo-A. Because the ELISA utilizes the central inhibitory domain of Nogo-A as the capture agent for 11c7, mimicking the *in vivo* mechanism, we propose that immunoreactive 11c7 is bioactive.

Based on this principle we developed an ELISA for the anti-Nogo-A 11c7 and demonstrated that the assay is both quantitative and a measure of bioactivity. To our knowledge, this is the first non-cellular assay to measure bioactive anti-Nogo-A 11c7: its primary benefits over earlier methods are speed, ease of use and quantitative results.

2. Description of methods

2.1. Development of the anti-Nogo-A ELISA

With reference to a publicly available ELISA development guide [22], grid experiments were used to evaluate a total of 16 analytical conditions in the following ranges: capture antibody, 0.25–4.0 $\mu\text{g}/\text{mL}$ (rat Nogo-A/human IgG Fc chimera, R&D Systems, Minneapolis, USA); analyte, 375–10,000 pg/mL (mouse anti-rat Nogo-A 11c7, Novartis, Basel, CH); and detection antibody, diluted 1:500–1:4000 (donkey anti-mouse IgG coupled to horse radish peroxidase, R&D Systems). The completed sandwich ELISAs were then incubated with tetramethylbenzidine and hydrogen peroxide (R&D Systems) and quenched with 1M sulfuric acid (Sigma–Aldrich, Oakville, CA). All assays were run in 96-well, high binding, flat bottom micro titer plates (Greiner Bio-One, Monroe, USA), read at 450 nm and optically blanked at 540 nm using a VersaMax microplate reader (Molecular Devices, Sunnyvale, USA).

All buffers were made with distilled and deionized water prepared using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 18 M Ω resistance (Millipore, Bedford, USA). Phosphate buffered saline powder was purchased from MP Biomedicals (pH 7.4, 9.55 g/L, Solon, USA); Tween 20, bovine serum albumin (BSA), and all other reagents were purchased from Sigma–Aldrich unless otherwise noted.

The grid experiments yielded multiple acceptable assay conditions (Fig. 1) and demonstrated the assay could be tuned to give larger linear ranges (shallower slope) or higher sensitivity (steeper slope). Under optimized conditions linear responses as long as 1000–6000 pg/mL or as short as 500–2000 pg/mL were obtained: the shorter range provided three fold better sensitivity. When used at dilutions of 1:1000 or lower, the detection antibody

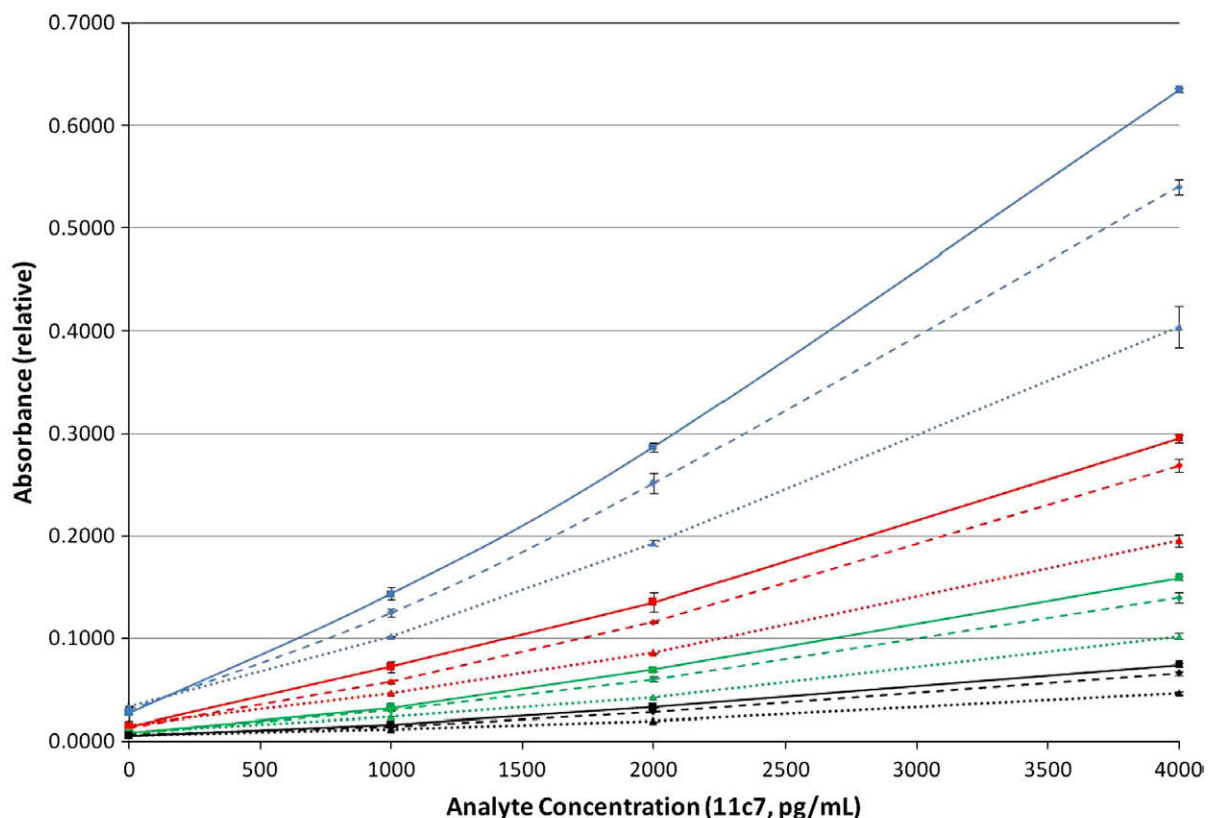


Fig. 1. Typical results from a grid experiment evaluating 12 ELISA conditions. Absorbance values have not been blanked in order to illustrate the inherent background. Color denotes detection Ab dilution: blue, 1:500; red, 1:1000; green, 1:2000; black, 1:4000. Line style denotes capture Ab concentration: solid, 1 $\mu\text{g}/\text{mL}$; dashed, 2 $\mu\text{g}/\text{mL}$; dotted, 4 $\mu\text{g}/\text{mL}$. The data are $n=2$, error bars are ± 1 standard deviation. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

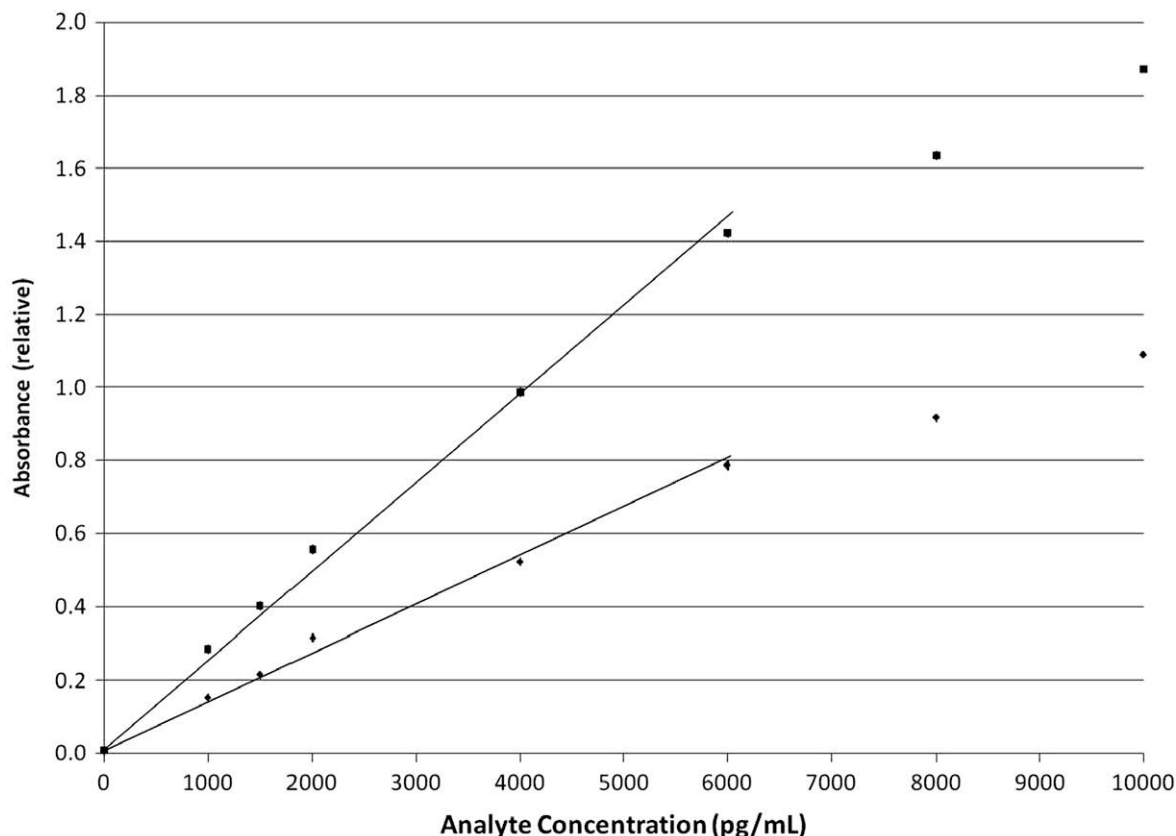


Fig. 2. Two selected standard curves collected under optimized conditions showing the range of sensitivities over the linear range of 1000–6000 pg/mL. Both curves are 1 µg/mL capture Ab: the detection Ab was diluted 1:500 in the upper curve, 1:1000 in the lower. The data are $n=3$, error bars are ± 1 standard deviation.

yielded vigorous color development and signal to noise ratios greater than 10 for all points within the ranges given (Fig. 2).

2.2. An optimized anti-Nogo-A ELISA protocol

The following protocol was optimized to yield linear calibration curves in the range of 1000–6000 pg/mL after a 20 min incubation of the substrate solution (the upper curve in Fig. 2). See the ELISA Development Guide [22] or other suitable reference for guidelines on proper technique. All incubations were conducted at 20 °C. Reagents and buffer compositions are fully described in Table 1.

1. Add 100 µL/well of the capture antibody, seal and incubate for 16 h.
2. Aspirate and wash with wash buffer five times (300 µL/well).
3. Add 300 µL/well of the blocking buffer, seal and incubate for 1 h.
4. Repeat Step 2.
5. During the blocking step, prepare serial dilutions of 11c7 standard in diluent buffer as desired. Dilute samples with diluent buffer. Standards and samples should be run in triplicate.

Table 1
Buffers and reagent solutions used in the optimized ELISA protocol

Component	Composition ^a
Capture antibody	1.0 µg/ml rat Nogo-A, freshly prepared
Wash buffer	1% v/v Tween 20
Blocking buffer	1% w/v BSA
Detection antibody	1:500 dilution of anti-mouse IgG conjugated HRP in diluent buffer
Diluent buffer	0.1% w/v BSA + 0.05% v/v Tween 20
Substrate solution	Tetramethylbenzidine and hydrogen peroxide prepared as per the manufacturer's instructions
Stop solution	1-M sulfuric acid in water

^a Prepared in 9.55 g/L PBS unless otherwise noted.

6. Add 100 µL of a standard or sample to each well, agitate gently, seal and incubate for 2 h.
7. Repeat Step 2.
8. Add 100 µL of the detection antibody to each well, seal and incubate for 2 h.
9. Repeat Step 2.
10. Add 100 µL of the substrate solution to each well, seal and incubate for 20 min.
11. Add 50 µL of the stop solution and agitate gently. Read the absorbance of the yellow product at 450 nm within 30 min. Correct for optical imperfections in the plate by subtracting the absorbance at 540 nm from the value at 450 nm, if required.

2.3. Preparation of 11c7 ELISA samples

Test solutions of 11c7 (1 mg/mL in PBS) were either boiled at 100 °C, exposed to 10% v/v dichloromethane (DCM) or to 10% v/v acetone. All exposures were 10 min followed by rapid freezing and storage at –20 °C until use. Control samples of fresh 11c7 and mouse IgG (Invitrogen, Carlsbad, USA) as a negative control, were subjected to the same freeze/thaw cycle. Samples were thawed immediately before use, diluted in diluent buffer (Table 1) and added to the ELISA plate as described above.

2.4. Circular dichroism spectroscopy

Circular dichroism spectroscopy (CD) detects the presence and relative arrangement of a protein's secondary structures through the absorption of circularly polarized light. CD spectra of fresh 11c7 and denatured 11c7 (concentration matched at 175 µg/mL) were obtained using an Aviv 202 CD Spectrometer (Aviv Associ-

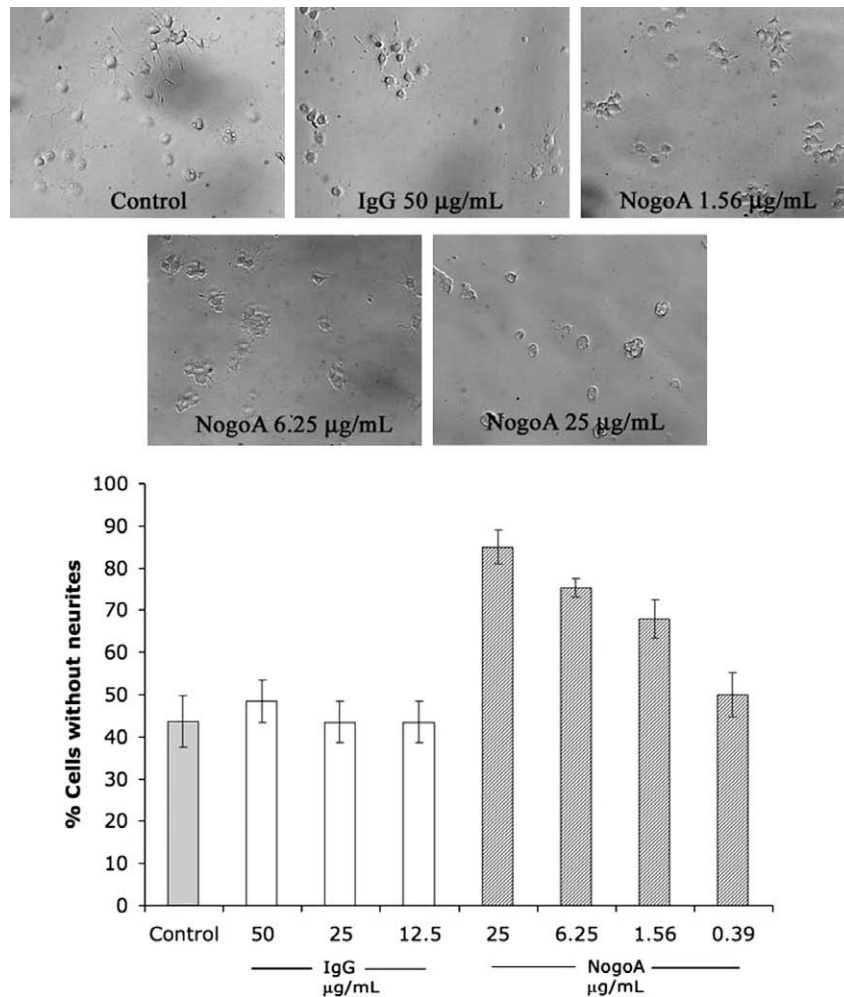


Fig. 3. Adsorption of a Nogo-A/Fc chimera significantly inhibits PC12 neurite outgrowth at concentrations similar to those reported for myelin protein extracts. The presence of a control IgG does not inhibit outgrowth, supporting the specific activity of Nogo-A.

ates, Lakewood, USA) at 25 °C and a path length of 1 mm. Samples were scanned from 200–260 nm in 2 nm increments with a 5 s read time and averaged over 10 measurements.

2.5. Routine cell culture

PC12 cells were grown in a controlled environment incubator at 37 °C and 5% CO₂ using high glucose DMEM (Sigma, St. Louis, USA) supplemented with 10% horse serum (Wisent Bioproducts St. Bruno, CA), 5% fetal bovine serum (Wisent Bioproducts), 100 U/mL penicillin (Invitrogen), and 0.5 mg/mL streptomycin (Invitrogen). Cells were passaged by mechanical detachment in Dulbecco's PBS (D-PBS; minus calcium and magnesium).

2.6. Neurite outgrowth assay

The cellular assay for neurite outgrowth was done as previously reported [21] with certain modifications. Each incubation prior to cells addition was conducted at 20 °C unless otherwise stated.

2.7. Coating growth surface

1. Add 150 µL of Nogo-A (R&D Systems), IgG (Sigma), or PBS to wells of an 8-well plastic slide (Nalge Nunc, Rochester, USA) for 2 h.
2. Aspirate and rinse twice with PBS.
3. Add 0.01% wt/vol poly-D-lysine and incubate for 30 min.

4. Aspirate and rinse twice with PBS. Immediately add primed PC12 cells (see below).

2.8. Priming PC12 cells

1. Prime cells with 100 ng/mL NGF (AbD Serotec, Kidlington, UK) in reduced serum DMEM (1% horse serum and 0.5% fetal bovine serum) for 2 days.
2. Aspirate media and add D-PBS for 5 min at 37 °C.
3. Mechanically detach cells with repeated application of the D-PBS.
4. Centrifuge the cells at 300g for 5 min.
5. Aspirate D-PBS and re-suspend cells in reduced serum DMEM.
6. Plate on plastic slides (treated with conditions outlined above) in low serum DMEM with 100 ng/mL NGF at a density of 4000 cells/cm².
7. Add 100 ng/mL NGF daily.

2.9. Analysis

1. After 3 days, image 5–6 randomly chosen fields per well using an inverted microscope at 20× magnification.
2. Alternatively, fix the cells with 4% paraformaldehyde in PBS by adding 200 µL per well for 30 min, rinse three times with PBS, and coverslip with a mounting medium such as Mowiol.
3. Count the number of cells with one or more neurites longer than the diameter of the cell body.

2.10. Statistical methods

All data presented graphically are the means of at least two trials and are shown as plus and minus one standard deviation. Results from cell assays were compiled from at least 150 cells/well taken from five randomly selected fields of view.

3. Results and discussion

In agreement with previous reports [18,21], PC12 cells extended neurites over poly-D-lysine coated substrates. This growth was inhibited in a dose dependant manner at 3 days by co-adsorption of a rat Nogo-A fragment bearing the central inhibitory domain of Nogo-A, at concentrations of 0.39–25 µg/mL. The percentage of cells without neurites increased from the control value of 43±6% to 85±4% in the presence of this fragment at 25 µg/mL (Fig. 3). This result is in good agreement with reports of neurite inhibition in the presence of 1–20 µg/well adsorbed myelin proteins, wherein the range from control to maximum inhibition was 28–70% [21]. To verify the observed inhibition was not due to competitive adsorption, whereby Nogo-A displaces the growth permissive poly-D-lysine, IgG was adsorbed at concentrations up to 50 µg/mL prior to poly-D-lysine treatment without an observed reduction in neurite outgrowth. Fig. 3 demonstrates that the Nogo-A/Fc fragment specifically inhibits PC12 neurite outgrowth in a manner consistent with that of the extracted myelin proteins used in cell assays [18,21]. This observation is supported by the known presence of Nogo-A in myelin [25] and justifies use of the Nogo-A/Fc fragment as a valid capture agent for the anti-Nogo-A 11c7. This result was expected, as the Nogo-A fragment contains the amino acid sequence against which 11c7 was raised. The effectiveness of this ELISA as an assay for bioactive 11c7 is therefore due to the use of a capture agent which presents the antigen for 11c7, replicating the binding interaction which is basis of the antibody's biological activity. Based on the currently known mechanism of anti-Nogo-A action proposed in the literature, these mAbs function by preventing binding to neuronal cell surface receptors (and subsequent intracellular signaling) [9–13]. If this is the only mechanism of action, immunoreactivity and bioactivity are synonymous because 11c7 binding to Nogo-A blocks cellular interaction with the protein, mitigating its growth inhibitory effects. When comparing the assays, both utilize Nogo-A to capture 11c7 from solution, an event detected in the cell assay by resumption of neurite extension and in the ELISA through binding to an anti-IgG-HRP conjugate. The adsorbed antigen, soluble antibody arrangement in the ELISA is atypical but directly mimics the biological mechanism. The robustness of the assay is increased because 11c7 acts on Nogo-A, a ligand for multiple cell surface receptors, not a receptor on cells of interest (e.g. neurons) where the quality of binding may affect intracellular signaling. Indeed, although 11c7 was raised against only one of the inhibitory regions of Nogo-A, it has been suggested the effectiveness of this IgG is partially due to simple steric blocking of the generalized Nogo-A-NgR interaction [14].

To confirm the ELISA detects only bioactive anti-Nogo-A, a series of 11c7 samples were exposed to various denaturing conditions and tested blind in the ELISA with fresh 11c7 and mouse IgG as positive and negative controls, respectively. By ELISA, fresh 11c7 tested at 100% of the expected value and mouse IgG gave no detectable signal. Heat treatment of 11c7 precipitated the protein

so samples were centrifuged at 21,000g (10 min) to yield a clear supernatant: this fully denatured sample also gave no detectable signal by ELISA or CD, the later method confirming the secondary structure of 11c7 had been destroyed. Exposure to DCM, a sparingly soluble organic solvent, reduced 11c7 immunoreactivity by over 50%. Interestingly, the CD signal of 11c7 decreased by over 30% when treated with 10% v/v acetone yet remained 100% immunoreactive by ELISA. This apparent anomaly may be attributable to solvent induced renaturation [26], because the denaturing agent concentration is reduced by a factor of one million during ELISA sample preparation. This mechanism is not applicable to the DCM sample, wherein 11c7 denaturation likely occurs at the aqueous-organic interface and there is minimal soluble DCM to dilute during ELISA sample preparation.

4. Conclusion

The current work reports the development and validation of a sandwich ELISA for detection and quantification of a bioactive anti-Nogo-A, the mAb 11c7. The ELISA utilizes the same antibody-antigen interaction which underpins a widely used cellular assay and yields consistent results for control and blinded samples. The state of 11c7 de/naturation was confirmed by circular dichroism spectroscopy and the substitution of a Nogo-A/Fc chimera for Nogo-A by PC12 cell assay. As an accurate measure of 11c7 bioactivity ELISA is quantitative, easy to use and faster than the current alternative-measuring in a day what previously required four.

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