

Better 'Insite' with ELISA In Situ



ELISA In Situ: A Sensitive Tool for Detecting Release of Endogenous BDNF

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Editor's Note: As of March 2001, we have improved the E_{max}[®] ImmunoAssay Systems. Please see the information at the end of this article (page 20) describing the changes and new catalog numbers for these assay systems.

Of note here, the Anti-IgY HRP Conjugate provided with the improved BDNF E_{max}[®] ImmunoAssay Systems (Cat.# G7610, G7611) should be diluted 1:50 instead of 1:1,000 to perform the ELISA in situ protocol described in this article.

Abstract

To measure release of endogenous BDNF from neurons, we developed an *in vitro* model using dissociate cultures of rat primary sensory ganglion cells and a modified ELISA, termed ELISA in situ (1). Nodose-petrosal ganglion (NPG) neurons were grown in 96 well ELISA plates precoated with Promega's Anti-BDNF mAb to capture released BDNF, which we subsequently detected using the antibody sandwich format of the Promega BDNF E_{max}[®] ImmunoAssay System (Cat.# G7611, G7610). Release was measured in response to either patterned electrical field stimulation or chronic depolarization with elevated extracellular potassium and compared with basal release in the absence of depolarizing stimuli. Incorporation of the coating antibody into the culture system dramatically increased detectability of BDNF in both control and stimulated cultures. The efficacy of the *in situ* assay appears to be related primarily to rapid capture of released BDNF.

Introduction

Recognition that brain-derived neurotrophic factor (BDNF) is critically involved in activity-dependent processes of synaptic development and plasticity has sparked growing interest in understanding the mechanisms that regulate neuronal release of neurotrophins (2). However, analysis of secretion of endogenous neurotrophins from identified neurons has been hampered by the limited ability of conventional assays to detect the relatively small quantities of these factors released during physiologic stimulation. Studies to date have used the enzyme-linked immunosorbent assay (ELISA) to detect neurotrophin release either from tissue slices or following neurotrophin overexpression in transfected cells (3–9). However, the effect of overexpression to very high concentrations on normal routes of BDNF trafficking and release is not known. Therefore, to study

release of endogenous BDNF from primary sensory neurons, we used a modification of conventional ELISA methodology, termed ELISA in situ. This technique, first described by Beech *et al.* (10) for measuring cytokine release from T-cells, incorporates a substrate-bound monoclonal antibody against the peptide of interest into the cell culture system so that the released peptide is immediately captured for subsequent detection by colorimetric methods. Using this technique, we can readily detect both constitutive and regulated release of endogenous BDNF from newborn primary sensory neurons with the Promega BDNF E_{max}[®] ImmunoAssay System.

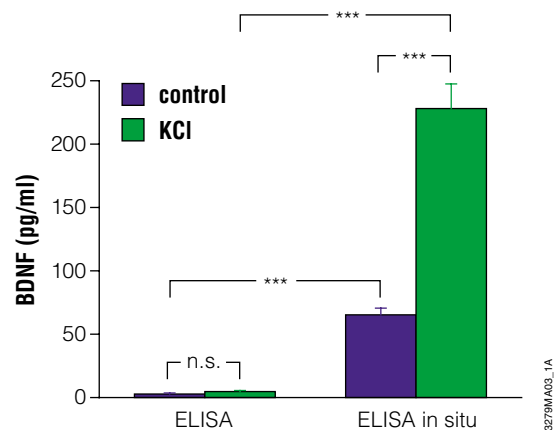


Figure 1. Extracellular levels of endogenous BDNF detected with standard ELISA and ELISA in situ. Mean BDNF levels were measured in sister cultures of newborn NPG neurons grown for 72 hours in the absence (control) or presence of depolarizing concentrations of potassium (40mM; KCl); n=21; *** P<0.001; n.s., not significant.

ELISA In Situ Detects Release of Endogenous BDNF Following Chronic Depolarization of Dissociated Cells

As illustrated by Figure 1, we were able to detect only very low levels of BDNF in control cultures and saw no significant change in BDNF concentration in KCl-treated groups compared to controls using a conventional BDNF ELISA protocol. On the other hand, we were able to measure higher levels of BDNF in control cultures and to detect significant release of BDNF following chronic depolarization using BDNF ELISA in situ. The increase in BDNF levels detected in KCl-treated cultures was not due

to increased neuronal survival ($1,234.3 \pm 71.05$ neurons/well in control cultures vs. $1,429.6 \pm 91.22$ neurons/well in KCl-treated cultures, $n=9$, $P=0.53294$). Similarly, survival was not significantly increased by the presence of the BDNF antibody during the cell culture period ($1,234.3 \pm 71.05$ neurons/well in the presence of anti-BDNF vs. $1,197.56 \pm 102.78$ neurons/well in the absence of anti-BDNF, $n=9$, $P=0.70989$).

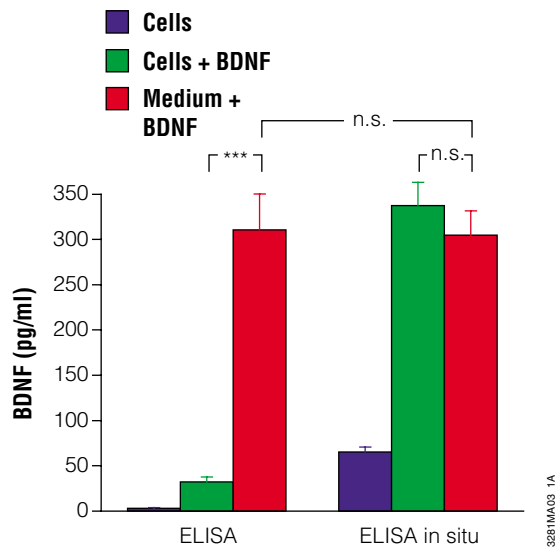


Figure 2. Detectability of exogenous BDNF by standard ELISA versus ELISA in situ. BDNF (Cat.# G1491; 500pg/ml) was added at plating to NPG cultures and to culture medium alone and incubated for 72 hours in the absence (standard ELISA) or presence of Anti-BDNF monoclonal capture antibody (ELISA in situ). BDNF levels were also measured in control cultures to which exogenous BDNF was not added. *** $P<0.001$; n.s., not significant.

To determine whether the presence of antibody by itself stimulated BDNF release, sister cultures were grown in wells precoated with an irrelevant immunoglobulin, Anti-NGF mAb, in the presence of absence of elevated KCl, and then assayed for BDNF using conventional BDNF ELISA. The presence of the antibody had no effect on BDNF levels, either in control or KCl-treated cultures. Together, these data demonstrate that the markedly higher levels of BDNF that were detected in control and stimulated cultures using ELISA in situ compared to conventional ELISA could not be attributed to increased neuronal survival or other potentially nonspecific effects of the in situ assay protocol.

Rapid Capture Prevents Released BDNF from Binding to TrkB Receptor

We hypothesized that the in situ assay protocol increased BDNF detectability by rapidly capturing and immobilizing released BDNF, preventing the peptide from binding to

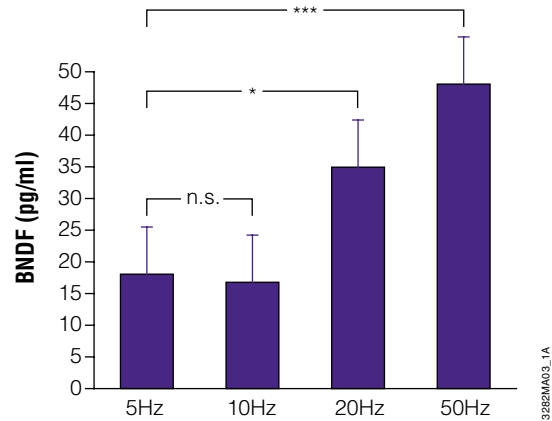


Figure 3. Activity-dependent release of BDNF is regulated by the pattern of stimulation. Mean levels of BDNF released from P0 NPG neurons during 60 minutes of electrical field stimulation with 50 biphasic rectangular pulses of 10ms, delivered at 5, 10, 20 and 50Hz, with interburst intervals, respectively, of 0, 10, 15 and 18 seconds. *** $P<0.001$, * $P<0.05$; n.s., not significant.

cells or protecting it from degradation. To test these possibilities, Human Recombinant BDNF (500pg/ml; Cat.# G1491) was added to culture wells containing either medium alone or NPG dissociate cultures. Following 72 hours of incubation, BDNF levels were compared in both groups using conventional ELISA and ELISA in situ. No significant differences were found between the levels of BDNF detected by standard ELISA and ELISA in situ (Figure 2) in wells containing culture medium alone plus BDNF. This experiment demonstrates that there are no intrinsic differences in the sensitivity of the two assays. However, when compared among wells containing NPG neurons plus BDNF, the difference between the levels of BDNF detected with the two assays was highly significant ($P=0.00047$). Specifically, using standard ELISA, the BDNF concentration was not significantly different from BDNF levels in medium from control NPG cultures grown with no added BDNF (Figure 2). This result indicates that, in the presence of NPG cells, BDNF is lost over time from the culture medium, perhaps through degradation or binding to the high-affinity BDNF receptor, TrkB, which is expressed by newborn NPG neurons (11). In fact, treatment of cultures with a function-blocking anti-TrkB antibody (Transduction Laboratories; for details see reference 1) significantly increased the ability of conventional ELISA to detect BDNF added to standard NPG cultures. These data suggest that the relative inability of conventional ELISA to detect BDNF release in NPG cultures is due, in large part, to binding of BDNF to TrkB on the cultured cells.

In contrast to the results obtained with standard ELISA, ELISA in situ detected levels of BDNF in the presence of cells that were not significantly different from those in wells

to which BDNF was added in the absence of cells (Figure 2). These data demonstrate that the substrate-bound anti-BDNF, which is present throughout the culture period in the in situ paradigm, successfully competes with BDNF binding to TrkB on cells, thereby enhancing detectability of BDNF in the culture medium.

Measurement of Changes in BDNF Release in Response to Electrical Stimulation

Given the sensitivity of the BDNF ELISA in situ technique, we next sought to compare levels of BDNF release in response to different patterns of electrical stimulation. To approach this issue, we used a paradigm in which the overall number of pulses, and consequently, average frequency, as well as the number of pulses in individual bursts, remained constant, whereas intraburst frequency and interburst interval were varied. Specifically, BDNF release was compared during 60 minutes of either control conditions or electrical field stimulation with 50 biphasic rectangular pulses of 10ms, delivered at 5, 10, 20 and 50Hz, with interburst intervals, respectively, of 0 (tonic stimulation), 10, 15 and 18 seconds. BDNF release was significantly higher during stimulation with high-frequency bursts (20Hz, 34.95 ± 4.98 pg/ml, $P=0.0144$; 50 Hz, 48.07 ± 7.18 pg/ml, $P=0.0005$) compared to tonic stimulation at 5Hz (18.09 ± 2.79 pg/ml, $n=10$; Figure 3). When compared among different bursting patterns, stimulation with 2-second 50Hz bursts delivered every 20 seconds was most effective, despite the short burst duration and long interburst interval characteristic of this pattern (Figure 3).

Conclusions

Our studies demonstrate that the detectability of secreted BDNF can be markedly enhanced by adoption of the Promega BDNF E_{max}[®] ImmunoAssay System to an ELISA in situ assay as described here. We have recently extended our use of this method to analyze BDNF release from cortical and hippocampal neurons and find it to be as effective as described here for analysis of sensory neurons in culture (Balkowiec and Katz, unpublished observations). By obviating the need to overexpress BDNF in order to detect release, the ELISA in situ technique makes it possible to characterize release properties under more physiological conditions than previously possible and should be applicable to analysis of other secreted neuronal proteins as well.

Methods

Cell culture: Cultures of NPG primary sensory neurons were obtained from newborn Sprague Dawley rats as previously described (1).

BDNF immunoassays: BDNF protein was measured with both a conventional and a modified sandwich ELISA using Promega BDNF E_{max}[®] ImmunoAssay System (Cat.# G6981; see Editor's Note on page 18) according to the protocol described in the *BDNF E_{max}[®] ImmunoAssay Technical Bulletin #TB257*, except that the concentration of the Anti-BDNF Monoclonal and Anti-Human BDNF Polyclonal Antibody were 5µg/ml and 2µg/ml, respectively, and the dilution of the anti-IgY-HRP antibody was 1:1,000 (see Editor's Note). All reagents used prior to cell plating were sterilized with 0.2µm Acrodisc[®] syringe filters (Pall).

Conventional BDNF ELISA: Cells were grown in uncoated, UV-sterilized, 96 well ELISA plates. In some control experiments, wells were precoated with an irrelevant monoclonal antibody (Anti-NGF mAb; Promega Cat.# G1131) to rule out any potential influence of antibody presence on BDNF release. These wells were treated prior to cell plating as described below for Anti-BDNF mAb. On the day of the assay, a standard curve was generated for each plate using BDNF diluted in the same medium used for cell culture. Standards (in duplicate) and undiluted fresh samples of cell-conditioned culture medium (in duplicate or triplicate) were incubated in ELISA plates precoated with Anti-BDNF mAb according to Technical Bulletin #TB257.

BDNF ELISA In Situ: 96 well ELISA plates were UV-sterilized for 30 minutes and coated with Anti-BDNF mAb at 4°C for 16.5 hours. Next, plates were washed and blocked, followed by two 1-hour incubations with culture medium to remove ELISA washing solution. NPG cells were plated in Anti-BDNF-coated wells and grown for three days under various experimental conditions. BDNF samples used to generate the standard curves were incubated in the same plate as the cells. At the end of the culture period, plates were extensively washed to remove all cells and cell debris, and the Anti-Human BDNF pAb was applied, followed by subsequent steps according to Technical Bulletin #TB257. In experiments designed to compare the conventional BDNF ELISA with BDNF ELISA in situ, all steps of the protocol, beginning with the application of the Anti-Human BDNF pAb, were performed simultaneously for both assays. Absorbance values were read at 450nm in a plate reader (Vmax[®], Molecular Devices). For control wells in which Anti-BDNF mAb was omitted, absorbance values were not significantly different from those of blank wells.

Calculations and statistical analysis: BDNF levels were calculated from the standard curve prepared for each plate using SOFTmax[®] PRO v. 3.0 software (Molecular Devices). The standard curves were linear within the range used (0–500pg/ml), and the quantities of BDNF in experimental samples were always within the linear range of the standard curve. Data are expressed as mean \pm standard error. Samples were compared using ANOVA followed by Duncan's multiple comparison procedure; $P<0.05$ was considered significant.

Acknowledgment

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References

1. Balkowiec, A. and Katz, D.M. (2000) *J. Neurosci.* **20**, 7417–7423.
2. McAllister, A.K., Katz, L.C. and Lo, D.C. (1999) *Ann. Rev. Neurosci.* **22**, 295–318.
3. Blöchl, A. and Thoenen, H. (1995) *Eur. J. Neurosci.* **7**, 1220–1228.
4. Blöchl, A. and Thoenen, H. (1996) *Mol. Cell. Neurosci.* **7**, 173–190.
5. Canossa, M. *et al.* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 13279–13286.
6. Goodman, L.J. *et al.* (1996) *Mol. Cell. Neurosci.* **7**, 222–238.
7. Griesbeck, O. *et al.* (1999) *Microsc. Res. Tech.* **45**, 262–275.
8. Heymach, J.V. *et al.* (1996) *J. Biol. Chem.* **271**, 25430–25437.
9. Krüttgen, A. *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95**, 9614–9619.
10. Beech, J.T., Bainbridge, T. and Thompson, S.J. (1997) *J. Immunol. Meth.* **205**, 163–168.
11. Zhuo, H. and Helke, C.J. (1996) *Brain Res. Mol. Brain Res.* **38**, 63–70.

Protocol

- ◆ *BDNF E_{max}[®] ImmunoAssay System Technical Bulletin #TB257*, Promega Corporation.
(www.promega.com/tbs/tb257/tb257.html)



Ordering Information

Product	Size	Cat.#	Price (\$)
BDNF E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7610	265
	5 × 96 wells	G7611	515
Recombinant Human BDNF	5µg	G1491	247
Anti-NGF mAb	100µg	G1131	357
	20µg	G1132	95

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IMPORTANT NOTICE

Regarding Improvements to the E_{max}[®] ImmunoAssay Systems

Technical Bulletins #TB196, #TB221, #TB224, #TB226, #TB243, #TB257, and #TB261 contain revised protocols for the E_{max}[®] ImmunoAssay Systems due to changes in the conjugate and TMB substrate. These changes will maintain the sensitivity, low background and range that you have come to expect from these systems while increasing shelf life.

The E_{max}[®] ImmunoAssay System changes include:

1. The catalog number for each system has changed, and a new 2 × 96 well TGFβ₁ E_{max}[®] ImmunoAssay System (Cat.# G7590) has been added.
2. The volume and dilution of the Conjugate has changed due to the addition of stabilizer.
3. The TMB substrate has changed from the two-component TMB Solution and Peroxidase Substrate to the single component, ready-to-use **TMB One Solution**.
4. The stop solution has changed from 1M Phosphoric acid to 1N Hydrochloric acid.
5. The storage conditions have changed from 4°C to –20°C, until first use, followed by storage at 4°C.
6. The product will be shipped on dry ice instead of gel ice.

Please discard any previous versions of E_{max}[®] ImmunoAssay System Technical Bulletins. The most recent versions of Promega's Technical Bulletins and Technical Manuals may be found on the Internet at www.promega.com/tbs/.

Please look to our web site or request a copy of any E_{max}[®] ImmunoAssay System Technical Bulletin for a full description of new volume and dilution values for the Conjugates.

The additional E_{max}[®] ImmunoAssay Systems include those listed here.

Ordering Information

Product	Size	Cat.#	Price (\$)
TGFβ ₁ E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7590	265
	5 × 96 wells	G7591	515
TGFβ ₂ E _{max} [®] ImmunoAssay Systems	5 × 96 wells	G7600	515
NGF E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7630	265
	5 × 96 wells	G7631	515
NT-3 E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7640	265
	5 × 96 wells	G7641	515
NT-4 E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7650	265
	5 × 96 wells	G7651	515
GDNF E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7620	265
	5 × 96 wells	G7621	515
BDNF E _{max} [®] ImmunoAssay Systems	2 × 96 wells	G7610	265
	5 × 96 wells	G7611	515