(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) **EP 3 030 905 B9**

(12) **CORRECTED NEW EUROPEAN PATENT SPECIFICATION**

(15) Correction information:
Corrected version no 1 (W1 B2)
Corrections, see
Description Paragraph(s) 12, 44, 45

(48) Corrigendum issued on:
03.04.2024 Bulletin 2024/14

(45) Date of publication and mention of the opposition decision:
10.01.2024 Bulletin 2024/02

(45) Mention of the grant of the patent:
04.10.2017 Bulletin 2017/40

(21) Application number: **14761444.0**

(22) Date of filing: **08.08.2014**

(51) International Patent Classification (IPC):
G01N 33/569 *(2006.01)*

(52) Cooperative Patent Classification (CPC):
C12Q 1/6897; G01N 21/6408; G01N 21/6428

(86) International application number:
PCT/US2014/050424

(87) International publication number:
WO 2015/021433 (12.02.2015 Gazette 2015/06)

(54) BOTULINUM TOXIN ASSAY WITH IMPROVED SENSITIVITY

BOTULINUMTOXIN-ASSAY MIT ERHÖHTER EMPFINDLICHKEIT

DOSAGE DE TOXINE BOTULIQUE À SENSIBILITÉ AMÉLIORÉE

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR

(30) Priority: **09.08.2013 US 201361864436 P**

(43) Date of publication of application:
15.06.2016 Bulletin 2016/24

(60) Divisional application:
17183975.6 / 3 270 162
20182832.4 / 3 742 165

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Description

[0001] This application claims the benefit of priority to United States Provisional Application No. 61/864,436 filed on August 9, 2013. This and all other extrinsic materials discussed herein are incorporated by reference in their entirety. Where a definition or use of a term in an incorporated reference is inconsistent or contrary to the definition of that term provided herein, the definition of that term provided herein applies and the definition of that term in the reference does not apply.

Field of The Invention

[0002] The field of the invention is protease assays related to *botulinum* toxins.

Background

[0003] *Botulinum* neurotoxins (BoNTs) are produced by *Clostridium botulinum,* and are among the most potent toxins known. These toxins are a well-recognized source of food poisoning, often resulting in serious harm or even death of the victims. There are seven structurally related *botulinum* neurotoxins or serotypes (BoNT/A-G), each of which is composed of a heavy chain (~100 KD) and a light chain (~50KD). The heavy chain mediates toxin entry into a target cell through receptor-mediated endocytosis. Once internalized, the light chain is translocated from the endosomal vesicle lumen into the cytosol, and acts as a zinc-dependent protease to cleave proteins that mediate vesicle-target membrane fusion ("substrate proteins").

[0004] These BoNT substrate proteins include plasma membrane protein syntaxin, peripheral membrane protein SNAP-25, and a vesicle membrane protein synaptobrevin (Syb). These proteins are collectively referred to as the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins. Cleavage of SNARE proteins blocks vesicle fusion with plasma membrane and abolishes neurotransmitter release at neuromuscular junction. Among the SNARE proteins, syntaxin and SNAP-25 usually reside on the target membrane and are thus referred to as t-SNAREs, while synaptobrevin is found exclusively with synaptic vesicles within the synapse and is called v-SNARE. Together, these three proteins form a complex that is thought to be the minimal machinery to mediate the fusion between vesicle membrane and plasma membrane. BoNT/A, E, and C cleave SNAP-25, BoNT/B, D, F, G cleave synaptobrevin (Syb), at single but different sites. BoNT/C also cleaves syntaxin in addition to SNAP-25.

[0005] Due to their threat as a source of food poisoning, and as bioterrorism weapons, there is a need to sensitively and speedily detect BoNTs. Currently, the most sensitive method to detect toxins is to perform toxicity assay in mice. Such methods, however, entail considerable expense and are subject to regulations related to animal testing.

[0006] As a result, there is a growing interest in developing alternatives to animal-based methods for BoNT characterization. An attractive alternative is the use of cell-based assays, which maintain the receptor-based internalization and subsequent cleavage of the BoNT molecule that is generally absent from conventional *in vitro* assays. Such cell-based assays utilize cells that express constructs that are responsive to the BoNT, in some instances utilizing Förster resonance energy transfer (FRET) and in other instances utilizing non-FRET methods to provide fluorescence useful for the detection and characterization of BoNTs. Examples can be found in United States Patent Application No. 2004/0,191,887 (to Chapman), United States Patent Application No. 2006/0,134,722 (to Chapman), United States Patent No. 7,208,285 (to Steward), United States Patent No. 7,183,066 (to Fernandez-Salas), and United States Patent Application No. 2011/0,033,866 (to Atapattu), each of which is incorporated herein by reference in their entirety. For some applications, however, the sensitivity of such cell-based methods can be lacking. For example, United States Patent Application No. 2006/0,134,722 (to Chapman) discloses that EC50 value of cell based FRET assay to detect BoNTs is in the ≥10 pM range.

[0007] International Patent Application No. WO 2014/060373 (to Eisele) reported enhancement of the sensitivity of cells to intoxication with *botulinum* toxin by allowing certain tumor cells that had been primed for differentiation into neuronal cells to differentiate in a low osmolarity differentiation media for several days to several weeks prior to exposure to the toxin. Sensitivity was determined by lysis of the treated cells followed by a Western blot method directed towards SNAP-25. The utility of Western blotting as a quantitative method is considered debatable, however, and no data demonstrating the statistical significance of the reported differences was provided.

[0008] Although some success has been demonstrated in applying FRET assays to detection of BoNTs, the sensitivity of FRET assay to BoNTs has been still undesirable for many purposes. As few as 40 nanograms of BoNT is a lethal dose for most people, and samples suspected to contain BoNTs are often are prior to application to the test process. It is, therefore, strongly desirable to have methods that detect low concentrations of BoNT.

Summary of the Invention

[0009] Methods are disclosed that provide for increased sensitivity in cell-based assays for *botulinum* toxins in accordance with claims 1 and 8. Cells are provided that express a reporting construct that is responsive to *botulinum* toxin. Temperature and media compositions are identified that provide increased sensitivity of the transfected cell response to *botulinum* toxin relative to conventional temperatures and media compositions.

[0010] One embodiment of the inventive concept is a

method of increasing the sensitivity of cell-based detection of a *botulinum* toxin (BoNT/A) by providing a transfected cell that expresses a hybrid protein, where the hybrid protein includes a reporter-containing portion (for example, at or near a terminus of the hybrid protein) and a cleavage site, where the cleavage site is cleaved by a *botulinum* toxin to release the reporter-containing portion (for example, a portion containing a fluorophore) from the remainder of the hybrid protein. Suitable cells include neuronal, neuroendocrine tumor, hybrid, and stem cells. In this process transfected cell is exposed to a temperature of 38.5 °C to 39.5 °C during exposure to the *botulinum* toxin, resulting in at least a two-fold increase in the sensitivity of the cell to *botulinum* toxin relative to the cell's sensitivity to *botulinum* toxin at 37°C. In some embodiments the sensitivity enhancement is selective for BoNT/A. Alternatively, in some embodiments the transfected cells are exposed to a pre-toxin temperature (for example, 38.5 °C to 39.5 °C) prior to exposure to *botulinum* toxin. In some embodiments the hybrid protein includes a second fluorophore in addition to a fluorophore of the reporter-containing portion, and such a second fluorophore can be located at a different terminus of the hybrid protein than that of the reporter-containing portion. In preferred embodiments such fluorophores are arranged so that no significant (i.e. ≤5%) energy transfer via Förster resonance energy occurs between the fluorophores.

[0011] According to claim 8 of the inventive concept is a method of increasing the sensitivity of cell-based detection of a *botulinum* toxin by providing, in a first media a sodium concentration greater than 65mM, a transfected cell that expresses a hybrid protein, where the hybrid protein includes a reporter-containing portion (for example, at or near a terminus of the hybrid protein) and a cleavage site, where the cleavage site is cleaved by a *botulinum* toxin to release the reporter-containing portion (for example, a portion containing a fluorophore) from the remainder of the hybrid protein. The cell is then transferred to a second media having a sodium concentration of less than 50 mM (for example, less than 45 mM) and is contacted with a *botulinum* toxin in the second media. In some embodiments the transfected cell is exposed to the *botulinum* toxin upon transfer to the second media (i.e. without pre-incubation in the second media). Thereafter a signal is obtained from the reporter-containing portion of the hybrid protein. In such a method the sensitivity of the transfected cell to *botulinum* toxin can be increased by a factor of 10 or more relative to a method where the cell is maintained in and exposed to *botulinum* toxin in the first media (i.e. a media with a sodium concentration of at least 65 mM). The sodium concentration in the second media can be reduced by a reduction in the sodium content of a neurobasal media, for example by reducing the concentration of sodium chloride and/or sodium bicarbonate. In some embodiments the first media, the second media, or both can be of physiological osmotic strength. In other embodiments, the first media, the second media, or both can have an osmotic strength that is less than that of physiological osmotic strength, for example 250 mOsm or less.

Brief Description of The Drawings

[0012]

FIGS. 1A, 1B, and 1C show the response of transfected cells to *botulinum* toxin and fragments thereof at different temperatures. Fig. 1A shows the response of transfected cells to *botulinum* holotoxin at different temperatures. Fig. 1B shows the response of transfected cells to *botulinum* holotoxin or to the light chain of *botulinum* toxin at different temperatures. Fig. 1C shows the response of transfected cells to *botulinum* holotoxin in the presence of the heavy chain of *botulinum* toxin at different temperatures.

FIG. 2 shows the response of transfected cells to *botulinum* toxin at different pre-toxin exposure (i.e. culture) temperatures and toxin exposure (i.e. assay) temperatures.

FIG. 3A and 3B show the effect of media ionic strength on the response of transfected cells to *botulinum* toxins at different temperatures. Fig. 3A shows the response of transfected cells in a media having an ionic strength of 270mM. Fig. 3B shows the response of transfected cells to *Botulinum* toxin in a media having an ionic strength of 250mM.

FIGS. 4A and 4B show the effects of elevated temperatures on the response of transfected cells to *botulinum* toxin and in the absence of *botulinum* toxin. Fig. 4A shows the response of transfected cells to *botulinum* toxin at temperatures up to 41 °C. Fig. 4B shows brightfield and fluorescence photomicrographs of the response of transfected cells to temperatures of up to 41° C in the absence of *botulinum* toxin.

FIG. 5 shows the effect of increased temperature on a cell-based assay for BoNT/ E toxin.

FIGS. 6A to 6C show the effect of media with reduced sodium concentration on cell-based assays for *botulinum* toxin. Fig. 6A shows the effect of the use of proprietary media with different concentrations of added NaCl. Fig. 6B shows photomicrographs of transfected cells exposed to custom culture media containing different concentrations of NaCl.

FIG. 7 shows the response of transfected cells to *botulinum* toxin in media with different sodium concentrations and at different time points following exposure to the toxin.

FIGS. 8A, 8B, and 8C show the result of exposure of transfected cells to *botulinum* toxin in media with reduced sodium content for different lengths of time prior to the restoration of normal sodium concentrations. Fig. 8A shows the effects when transfected cell are cultured in conventional media prior to exposure to *botulinum* toxin. Fig. 8B shows the effects when the transfected cells are cultured in low sodium content media prior to exposure to *botulinum* toxin.

FIGS. 9A and 9B show the result of reducing sodium bicarbonate concentration in media used in cell-based assays for *botulinum* toxin. Fig. 8A shows dose/response curves in media with different concentration of sodium bicarbonate. Fig. 8B shows the effect on EC50s calculated from such dose/response curves as a function of sodium bicarbonate concentration in the media. Fig. 8C shows the effect of pre-incubation with either conventional media or media with reduced sodium content prior to the performance of a cell-based assay for *botulinum* toxin in low sodium content media.

FIG. 10 shows the effect of replacing sodium with potassium in custom media used in cell-based assays for *botulinum* toxin, and in the reduction of sodium and potassium concentrations in those media.

FIG 11 shows the effect of increasing the osmolarity of low sodium content media used in cell-based assays for *botulinum* toxin.

FIGS. 12A and 12B show the effects of *botulinum* toxin fragments and intact *botulinum* holotoxin on transfected cells in media with reduced sodium content. Fig. 12A shows the effect of adding recombinant *botulinum* toxin heavy chain to the transfected cells prior to exposure to the holotoxin. Fig. 12B shows the effect of adding recombinant *botulinum* toxin light chains to the transfected cells and the effect of adding the intact *botulinum* holotoxin to the transfected cells.

Detailed Description

[0013] The inventive subject matter provides methods in accordance with claim 8 in which the sodium ion concentration of cell culture media utilized in a cell-based *botulinum* toxin assay and/or the temperature at which the cell-based *botulinum* assay is performed is used to provide *botulinum* assays with enhanced sensitivity. Surprisingly, the inventors have found that reduction of sodium ion concentration in the cell culture media enhances sensitivity of *botulinum* toxin in an ion and *botulinum* toxin-specific manner. The inventors also identified a narrow range of temperatures over which the sensitivity of such assays, as defined by dose/response curves, is dramatically enhanced.

[0014] The inventive subject matter provides methods for improving the sensitivity of cell-based methods for detecting the presence of a *botulinum* toxin (BoNT) in accordance with claims 1 and 8. A variety of assays for *botulinum* toxins that utilize transfected cells expressing detecting constructs cleavable by these proteases have been developed. In cell-based assays, specific binding of the heavy chain of a *botulinum* toxin by cell surface receptors and followed by specific cleavage of a construct that includes a *botulinum* toxin-specific cleavage site by the light chain of the *botulinum* toxin and subsequent release of an indicator moiety (for example, a fluorescent protein) from the construct provide a high level of specificity. Prior art methods, however, lacked the sensitivity necessary for important applications of such assays, for example environmental testing. This is particularly important considering the potential use of *botulinum* toxins as bioweapons.

[0015] Unless the context dictates the contrary, all ranges set forth herein should be interpreted as being inclusive of their endpoints, and open-ended ranges should be interpreted to include only commercially practical values. Similarly, all lists of values should be considered as inclusive of intermediate values unless the context indicates the contrary.

[0016] Cell-based assays require a rigidly controlled environment, utilizing physiological ion concentrations, osmolarity (i.e. 250-270 mOsm), and temperatures held at physiological level. Surprisingly, the inventors have found that reducing the sodium ion concentration of the culture media provides a dramatic increase in the sensitivity of some cell-based BoNT assays. The effect is not seen with potassium ions, is not a result of changes in osmolarity of the cell culture media, and is not observed with certain types of *botulinum* toxins. The inventors have also found that elevating the temperature at which the assay is performed by up to 4 °C results in a dramatic increase in the sensitivity of such an assay without impacting cell viability over the course of the assay. Similarly, decreasing the osmolarity of the cell media also resulted in an increase in sensitivity. These methods can be adopted without the need for specialized equipment, and the increased sensitivity realized broadens the range of applications for these highly specific *botulinum* toxin assays.

[0017] Methods of the inventive concept provide a transfected cell, which in turn produces a construct or fusion protein. With respect to the transfected cells expressing the hybrid protein it is generally preferred that the cell is stably transfected. Nevertheless, transient transfection is also contemplated. It is still further typically preferred that the transfected cell is a neuronal cell. However, numerous other non-neuronal cells (including mammalian cells, insect cells, yeast, bacteria, and artificial cells) are also contemplated herein. Most typically, the cells will constitutively express the hybrid protein(s) are therefore under appropriate regulatory elements. In alternative aspects, the expression can also be induced.

[0018] Many choices of cell lines are suitable as the host cell for the present invention. Preferably, the cell is of a type in which the respective *botulinum* toxin (BoNT) exhibits its toxic activities. In other words, the cells preferably display suitable cell surface receptors, or otherwise allow the toxin to be translocated into the cell sufficiently efficiently, and allow the toxin to cleave the suitable substrate polypeptide. Specific examples include primary cultured neurons (e.g., cortical neurons, hippocampal neurons, spinal cord motor neurons, etc); PC12 cells or cell lines derived from PC12 cells; primary cultured chromaffin cells; cultured neuroblastoma cell lines (such as murine cholinergic Neuro2A cell line), human adrenergic SK-N-SH cell lines, NS-26 cell lines, and stem cells (see e.g. Foster and Stringer (1999), Genetic Regulatory Elements Introduced Into Neural Stem and Progenitor Cell Populations, Brain Pathology 9: 547-567). Similarly, neuroendocrine and neuroendocrine-derived cell lines can be used. It should be appreciated, however, that in the instance of recombinant or mutated BoNTs that are directed towards non-neuronal cell types, that host cells can be selected from cell lines with the corresponding specificity.

[0019] Constructs or fusion proteins of the inventive concept can include a reporter-containing portion and a cleavage site. The cleavage site can act as a substrate for the protease activity associated with a *botulinum* toxin light chain. Such transfected cells can demonstrate stable transformation or transient transformation. Cleavage of the cleavage site releases at least a portion of the reporter-containing portion from a remainder of the construct. The reporter region can include an observable reporting group or tag, such as a fluorophore which provides an observable fluorescence. Suitable fluorophores include fluorescent dyes, and can include fluorescent proteins such as Green Fluorescent Protein (GFP), Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP), Citrine, Venus, YPet, mStrawberry, and/or mCherry protein. In some embodiments the hybrid protein can include multiple fluorophores, for example a second fluorophore. Such a second fluorophore can be located within the reporter region or at a distal location. For example, the fluorophore of the reporter region (i.e. the first fluorophore) can be located proximate to one terminus of the hybrid protein while the second fluorophore can be located proximate to a different terminus of the hybrid protein. Alternatively, both the first fluorophore and the second fluorophore may be within the reporter region. Depending upon the nature of the detection, the first fluorophore and the second fluorophore can be the same fluorophore species, or can be different fluorophore species. For example, in an assay system utilizing FRET detection the first fluorophore and the second fluorophore can be different fluorophore species.

[0020] In a preferred embodiment of the inventive concept, the reporter region includes one or more fluorophores of the same species, which can be arranged so that homo-FRET does not occur to a significant degree (i.e. less than 5% Förster resonance energy transfer). In other embodiments the construct can include fluorophores of different species, which can be arranged so that FRET does not occur to a significant degree (i.e. less than 5% Förster resonance energy transfer). This can be accomplished, for example, by placing the fluorophores at or near different termini of the construct. In such embodiments the emission spectra of a first fluorophore can overlap with the excitation spectra of a second fluorophore without significant (i.e. less than 5%) Förster resonance energy transfer, however fluorescence emission of the first fluorophore is not significantly decreased (i.e. less than 5%) via quenching and fluorescence emission of the second fluorophore is not significantly (i.e. more than 5%) increased via such energy transfer. In other embodiments of the inventive concept the construct can include a first fluorophore with an emission spectrum that overlaps the excitation spectrum of a second fluorophore, with position of the fluorophores within the construct arranged such that significant (i.e. >5% Förster resonance energy transfer) occurs between the fluorophores. Fluorescence from a construct of the inventive concept can be detected by any means suitable for the configuration of the construct, for example including direct excitation and emission from each fluorescent species, FRET, and fluorescence anisotropy. In a preferred embodiment, a conventional microwell plate fluorometer configured for direct excitation and emission detection from each fluorophore species can be used.

[0021] Green fluorescent protein and its mutations, which fluoresce without the need for additional cofactors or substrates, are particularly suitable for use with constructs of the inventive concept. For example, Yellow Fluorescent Protein (YFP) is a mutation of the Green Fluorescent Protein, derived from *Aequorea victoria*, and has an excitation peak at 514nm and an emission peak at 527nm. In addition to YFP, it is also contemplated to use related Citrine, Venus, and YPet proteins can be used in the reporter-containing portion. These mutations have reduced chloride sensitivity, faster maturation, and increased brightness (product of the extinction coefficient and quantum yield) relative to GFP. Of course, any of the fluorescent proteins mentioned herein can be modified to include specific characteristics (e.g., spectral) or be truncated to a specific size. It is also contemplated that the reporter containing portion includes reporters other than fluorescent proteins (e.g., a phosphorescent compound, a luminescent compound, a chromophore, an enzyme, etc.).

[0022] In some embodiments of the inventive concept the detection signal is characterized prior to exposure of the transfected cells to the *botulinum* toxin (BoNT), to provide a baseline signal. This baseline signal can serve as a basis for comparison to an assay signal obtained following exposure of the transfected cells to *botulinum* toxin, and can serve to normalize such an assay signal to at least partially correct for variations in cell number, density, and/or shape between different test sites. For

example, the use of a ratio between a post-exposure signal and the baseline signal can serve to normalize fluorescence intensity between assays performed in different wells of a microwell plate, thereby reducing the variation between like measurements. Sensitivity can be assessed by preparing a series of such assays utilizing different concentrations of *botulinum* toxin to generate a dose/response curve, which is typically sigmoidal. Sensitivity can be quantified by determining the concentration of *botulinum* toxin that generates a response that correlates to a defined portion of the dose response curve. For example, a *botulinum* concentration that correlates with the midpoint or half-maximal value of the dose/response curve (typically reported as the EC50) can be used as a basis for comparing sensitivity in such assays.

[0023] Many different methods can be used to measure sensitivity to *botulinum* toxin using a cell-based assay. In one embodiment, an emission ratio of a first fluorescent protein and a second fluorescent protein that do not form a FRET pair (i.e. demonstrate less than about 5% energy transfer via FRET) can be measured after exposing the transfected cell to *botulinum* toxin. In such an embodiment, prior to exposure of the hybrid to *Botulinum* toxin, the construct exhibits a baseline signal, and the first fluorescent protein emission and the second fluorescent emission are separately measured. After exposure to *botulinum* toxin, the reporter-containing portion comprising the first fluorescent protein is cleaved by the *botulinum* toxin, and the cleaved reporter-containing portion is subsequently degraded by proteolysis. In such an example the emission intensity of the first fluorescent protein is decreased, while an emission intensity of the second fluorescent protein remains essentially the same. The emission measured from this second fluorescent protein is therefore a function of cell number, density, distribution, and so on, and is not a function of the concentration of *botulinum* toxin. As such, the emission from the second fluorescent protein can be used to normalize the emission measured from a fluorophore of the reporter region (in this instance the first fluorescent protein), for example by using an emission ratio. It should be appreciated that such an emission ratio is ineffective for data normalization in constructs in which the fluorophores are arranged to perform FRET, as the emissions from both fluorophores would change on cleavage of such a construct. The emission ratio (first fluorescent protein emission/ second fluorescent protein emission) is decreased when the construct interacts with *botulinum* toxin. An example of a suitable construct in such an embodiment is one that includes Cyan Fluorescent Protein (CFP) outside of the reporter region and in which the reporter region includes Yellow Fluorescent Protein (YFP), configured such that the CFP and YFP do not form a FRET pair. Data related to the degree of YFP degradation (i.e. directly, separately excited YFP emissions and CFP emissions) following exposure to a *botulinum* toxin can be collected from a cell expressing such a construct. Those emissions can be background subtracted and the YFP emission divided by the CFP emission to control for cell density and reporter expression in the individual cells.

[0024] *Botulinum* toxin responsive emission from a fluorophore of a reporter region or an emission ratio can be used to generate a dose response curve that is useful in quantifying *botulinum* toxin in a sample and/or to determine sensitivity of an assay to *botulinum* toxin. Such sensitivity is frequently expressed as a concentration of the BoNT corresponding to a characteristic portion of the dose/response curve. For example, a BoNT concentration corresponding to the midpoint of such a curve is referred to as an EC_{50}.

[0025] In one embodiment of the inventive concept, the transfected cells are exposed to the *botulinum* toxin at a temperature that is elevated relative to that at which cell culture and such assays are normally performed (i.e. 37.0 °C). It should be appreciated that such temperatures are generally considered non-optimal for cell survival, and that their use is counterintuitive in assays that rely on the use of viable cells. In a preferred embodiment the temperature at which the transfected cells are exposed to *botulinum* toxin is such that the sensitivity is increased at least two-fold (i.e. by a factor of 2) relative to an assay performed at 37.0 °C (i.e. the EC50 of the assay performed at the elevated temperature is less than half of the EC50 of the assay performed at 37.0 °C). Surprisingly, the inventors have found that such a sensitivity enhancement occurs within a relatively narrow range of temperatures. In accord with claim 1 the transfected cells are exposed to the *botulinum* toxin at 38.5 °C and 39.5 °C.

[0026] Alternatively, the transfected cells of the inventive concept can be maintained at temperatures greater than 37.0 °C prior to exposure to the *botulinum* toxin, 38.5 °C and 39.5 °C. In such embodiments, exposure of the transfected cells to *botulinum* toxin can be performed at 37.0 °C. Alternatively, in some embodiments transfected cells can be exposed to temperatures greater than 37.0 °C (38.5 °C and 39.5 °C) both prior to and during exposure to *botulinum* toxin.

[0027] In embodiments of the inventive concept, a cell-based assay detecting the presence of *botulinum* toxin can have an increase of at least two fold in sensitivity to *botulinum* toxin with a change of conditions (e.g., temperature, osmolarity, extracellular ion concentration, etc.). According to the present invention the sensitivity to *botulinum* toxin is increased at least three fold when the transfected cell is exposed to *botulinum* toxin at a higher temperature than 37.0 °C, within a range of 38.5 °C to 39.5 °C compared to sensitivity to *botulinum* toxin at 37.0 °C. In another embodiment the sensitivity is increased at least five fold when the transfected cell is exposed to *botulinum* toxin at a higher temperature than 37.0 °C, within a range of 38.5 °C to 39.5 °C compared to sensitivity to *botulinum* toxin at 37.0 °C. In still other embodiments the sensitivity is increased at least ten fold when the transfected cell is exposed to *botulinum* toxin at a higher temperature than 37.0 °C, within a range of 38.5 °C to 39.5 °C compared to sensitivity to *botulinum*

toxin at 37.0 °C.

[0028] It is also thought that reduced osmolarity of a cell media in which the transfected cell is exposed to BoNT can also enhance sensitivity to BoNT. Without wishing to be bound by theory, the inventors believe that reduced extracellular osmolarity can result in a modulation of cellular activity (e.g. neuronal excitability). Especially, in neuronal cells, reduced osmolarity enhances synaptic transmission and neuronal excitability, which increases the endocytosis rate. Thus, it is contemplated that a reduction in osmolarity of a cell media in which the transfected cell is exposed to *botulinum* toxin, for example to a range between 220 milliOsm and 260 milliOsm may confer an increase in sensitivity to *botulinum* toxin. It should be appreciated that such a modification is counterintuitive, as such conditions can adversely affect cell viability. Furthermore, it is also contemplated that reduced osmolarity of the cell media can enhance, for example in a synergistic fashion, an increased sensitivity to *botulinum* toxin that results from a higher temperature than 37.0 °C.

[0029] In another embodiment, increased sensitivity to *botulinum* toxin can be achieved by decreasing the concentration of specific extracellular ions, for example free (i.e. uncomplexed or non-chelated) calcium. When extracellular calcium concentration falls below normal physiological level, the transfected cell can be progressively more excitable. Similar to reduced osmolarity, increased cell excitability can enhance endocytosis rate and thus enhance the internalization of an applied *botulinum* toxin. Thus, it is also contemplated that reducing calcium concentration below the physiological level (1.0 - 1.5 mM) may confer a similar increase in sensitivity to *botulinum* toxin. Similarly, addition of calcium chelators (e.g., ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA/AM), and other organic acids) to the cell media may confer similar increases in sensitivity to *botulinum* toxin.

[0030] In accord with claim 8 an increase in the sensitivity of a cell-based assay for a *botulinum* toxin increased when the sodium ion concentration of the cell culture media utilized during the performance of the assay is reduced. For example, cell culture media can be prepared with sodium salts, for example NaCl and/or $NaHCO_3$, omitted from the formulation. Such cell culture media can have final sodium ion concentration of less about 50 mM, about 40 mM, about 30 mM, about 25 mM, or about 20 mM. In a preferred embodiment of the inventive concept the cell culture media used to perform a cell-based *botulinum* toxin assay is less than or equal to about 20 mM.

[0031] In performance of a cell-based assay, cells expressing a *botulinum* -sensitive construct as described above can be pre-incubated with a low sodium ion content culture media prior to exposure to the *botulinum* toxin, then contacted with a low sodium ion content culture media containing *botulinum* toxin (for example, from an added sample). In preferred embodiments of the invention, the cells are not exposed to a low sodium ion content culture medium prior to exposure of the cells beyond a brief (i.e. several minute) exchange or wash with low sodium ion content culture media prior to contact with the *botulinum* toxin.

[0032] In some embodiments of the inventive concept sodium ions in the cell culture media can be replaced by other ions that do not show the sodium ion effect (for example, potassium ions) or by other osmolarity modifying agents (for example, triethylamine N-oxide) to retain the physiological osmolarity of the cell culture media while still providing the sensitivity enhancement realized by the reduction in sodium ion concentration.

[0033] It should be appreciated that elevated temperature, reduced media osmolarity, reduced extracellular concentration of specific ions (for example, sodium ions), and additional protein can be combined, and that such combinations can exert a synergistic effect. For example, the inventors have surprisingly found that elevated temperature during exposure of the transfected cells to *botulinum* toxin and the use of media with reduced osmolarity has a synergistic effect on the improvement in sensitivity of a cell based assay.

[0034] A number of serotypes of *botulinum* toxin (BoNT) with different substrate specificities and specific cleavage sites have been identified, including BoNT/A, BoNT/B, BoNT/C, BoNT/D, BoNT/D, BoNT/E, BoNT/F, BoNT/C, and a proposed BoNT/H. In accordance with claims 1 and 8 a sensitivity enhancing method selective for BoNT/A. For example, the use of a low sodium content cell culture medium results in an enhanced sensitivity for a cell-based assay for BoNT/A, but have little effect on the sensitivity of a cell-based assay BoNT/E. In some embodiments of the inventive concept this selective enhancement of the sensitivity to one or more BoNT species occurs when the same cell line expressing the same construct is used in characterizing multiple BoNT species.

[0035] According to the present invention a construct of the inventive concept is responsive (i.e. act as a substrate) for BoNT/A. Similarly, it is contemplated that transfected cells expressing hybrid reporter/cleavage site bearing proteins that can act as substrates for recombinant or modified BoNT/A with altered specificity.

[0036] In a preferred embodiment, temperatures higher than 37.0 °C significantly and cell culture media with low (i.e. less than about 70 mM) sodium ion concentration enhance the sensitivity of the BOCELL™ model cell line to *botulinum* neurotoxin type A (BoNT/A). Such a cell line is described WO 2012/166943. All other extrinsic materials discussed herein are similarly incorporated by reference in their entirety.

[0037] BoNTs recognize the cleavage site and cleave the hybrid protein into the reporter-containing portion and the remainder of the hybrid protein. The cleavage site sequence of the present invention can advantageously comprise (a) a SNARE protein, motif, or mutein (or a cleavable portion of these). SNARE proteins are under-

stood to include SNAP-25, synaptobrevin (VAMP), and syntaxin. "Muteins" of a protein should be interpreted herein as having at least 30% identity with a corresponding native protein, including for example compositions having at least 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identity with the native protein. Variations from identity can comprise any or more of additions, deletions and substitutions. Contemplated muteins include fragments, truncates and fusion proteins.

[0038] Without wishing to be bound by theory, the inventors believe that the observed increased sensitivity to *botulinum* toxin at higher temperatures could be a consequence of increased specific binding and endocytosis of *botulinum* toxin. In the cell-based assay, BoNT must be internalized to the cell cytoplasm via receptor-mediated endocytosis. It is therefore possible that anything that causes more BoNT to be internalized, and to interact with the cleavage site of hybrid proteins/constructs, would result in the transfected cell being more sensitive to BoNT. As shown **Figure1A,** which depicts dose/response curves obtained with *botulinum* toxin at different temperatures, relatively small changes in temperature produce a surprisingly large effect on the sensitivity of a *Botulinum* toxin assay (as determined by EC50). **Figure 1B** shows the result of similar studies performed using intact *Botulinum* toxin (i.e. holotoxin) and the *botulinum* toxin light chain, which has protease activity capable of cleaving the cell's reporting construct but does not have receptor-binding activity. The lack of change in the emission ratio of the construct noted at conventional and elevated temperatures when the cells are exposed to the light chain indicates that temperature effects are not a result of generally enhanced endocytosis and are a result of receptor-mediated processes. This is supported by the data shown in **Figure 1C,** which shows the effects of different temperatures on transfected cells exposed to *botulinum* toxin in the presence of absence of *botulinum* toxin heavy chain, which lacks the ability to cleave the reporting construct but can occupy toxin-specific receptor sites. **Figure 1C** shows that the *Botulinum* toxin heavy chain is effective in blocking the effects of the holotoxin but less effective at the elevated temperature, indicating that the temperature effect of cell-based assay sensitivity may be a receptor-mediated process.

[0039] Increased expression of *botulinum* toxin receptor proteins on the transfected cell's surface may results in enhanced endocytosis of *botulinum* toxin. For example, *botulinum* toxin A, D and E are internalized to cell cytoplasm via interaction with synaptic vesicle proteins (SV2) expressed on cell surface. Thus, it is also contemplated that co-expression of SV2 protein in the transfected cell may infer similar increase in sensitivity to *botulinum* toxin.

[0040] Increased activity of endogenous stress response proteins, including Heat Shock Protein 70 (HSP70) and Heat Shock Protein 90 (HSP90), at the higher temperature can potentially induce enhanced sensitivity to *botulinum* toxin. Both HSP70 and HSP 90 are activated at higher temperatures than physiological temperature range (between 35.0 - 37.0 °C), and enhance proteolysis activity of the cell. Without wishing to be bound by theory, it is contemplated that increased activity of HSP70 or HSP90 can facilitate breakdown of the reporter-containing portion of the hybrid protein.

[0041] Still further, a conformational change of the hybrid protein at the higher temperature, by which the baseline FRET signal can be augmented, can induce enhanced sensitivity to *botulinum* toxin. HSP70 functions to aid proper folding of proteins, and increased activity of HSP70 may induce a conformational change of the hybrid protein. Therefore, it is also contemplated that treatment of HSP70 activator (e.g., YM1 (2-((Z)-((E)-3-ethyl-5-(3-methylbenzo[d]thiazol-2(3H)-ylidene)-4-oxothiazolidin-2-ylidene)methyl)-1-methylpyridin-1-ium chloride, 2-[3-Ethyl-5-(3-methyl-3H-benzothiazol-2-ylidene)-4-oxo-thiazolidin-2-ylidenemethyl]-1-methyl-pyridinium chloride)) to the transfected cell may infer similar increase in sensitivity to *botulinum* toxin.

[0042] It is further contemplated that various conditions described above can be combined to render further enhanced sensitivity to BoNT in the cell based assay. For example, reduced osmotic strength and reduced sodium concentration in the media can be combined to provide further sensitivity enhancements. It is contemplated that such combinations can produce synergistic effects.

EXAMPLES

[0043] ***Temperature Effects.*** Cell based assays to detect *botulinum* toxin (BOCELL™ assay) were performed at 35.0 °C, 37.0 °C, and 39.0 °C (Trials 1 and 2), and at 37 °C, 39 °C, and 41 °C (Trial 3) using *botulinum* toxin/A holotoxin at concentrations ranging between 10^{-15} M to 10^{-9} M. The transfected cells were exposed to one of the three temperatures while being exposed to the *botulinum* toxin. Dose/response curves were generated by characterizing emission ratios (YFP/CFP) at each concentration and plotting them as a function of *botulinum* toxin/A concentration. As shown in **Figure 1A,** by increasing the temperature used in the assay from 37 °C to 39.0 °C or 41 °C the sensitivity to *botulinum* toxin (measured as EC_{50} value) is enhanced more than 5 fold.

[0044] In the studies depicted in **Figure 1B** the transfected cells were treated with either *botulinum* toxin /A holotoxin or *botulinum* toxin /A light chain and incubated at either 37.0 °C or 39.0 °C. *Botulinum* toxin /A light chain retains the ability to cleave the detection construct expressed by the cells, but lacks the ability to bind to the specific cell surface receptor utilized by the intact holotoxin. In these studies *botulinum* toxin/A light chain does not cleave the cleavage site containing portion of the reporting construct, even at high concentrations. This indicates that intact BoNT/A undergoes receptor-mediated toxin uptake process and activation within the cell at both 37.0 °C and 39.0 °C, and that the enhanced sensitivity

is a receptor-mediated process.

[0045] Confirmation of this is found in the studies shown in **Figure 1C.** Transfected cells were pre-treated with *botulinum* toxin /A heavy chain or the equivalent vehicle prior to addition of *botulinum* toxin /A holotoxin. *Botulinum* toxin/A heavy chain lacks toxicity (i.e. proteoplytic activity) and cannot cleave the detecting construct expressed by the cell, but binds to an occupies the specific receptor bound by the holoxin. Preincubation of the transfected cells with the heavy chain, which comprises the receptor binding domain, *botulinum* toxin /A holotoxin uptake and reporter cleavage at both 37.0 °C and 39.0 °C, indicating a requirement for receptor-mediated endocytosis of BoNT/A holotoxin for reporter cleavage at elevated temperatires.

[0046] The effects of pre-treatment of cells using elevated temperatures is shown in **Figure 2.** Cell based assays to detect BoNT (BoCell™ assay) were performed at 35.0 °C, 37.0 °C, and 39.0 °C, using *botulinum* toxin /A holotoxin at concentrations ranging between 10^{-15} M to 10^{-9} M. The transfected cells were exposed to one of these three temperatures before being exposed to *botulinum* toxin, then exposed to the same or a different temperature among the three temperatures during exposure. The sensitivity to *botulinum* toxin, characterized as a reduced EC50 value, was enhanced in transfected cells exposed to *botulinum* toxin at 39.0 °C. Sensitivity to *botulinum* toxin at 39.0 °C was at least 3 fold greater than the sensitivity to *botulinum* toxin at 37.0 °C, and at least more than 10 fold compared to sensitivity to *Botulinum* toxin at 35.0 °C.

[0047] The effects of elevated temperature combined with reduced osmolarity are shown in **Figures 3A and 3B.** Cell based assays to detect *botulinum* toxin (BOCELL™ assay) were performed at 35.0 °C, 37.0 °C, and 39.0 °C, where the transfected cells were exposed to *Botulinum* toxin in a cell media with an osmolarity of approximately 270 mOsm (i.e. normal osmolarity). Consistent with previous observations and as shown in **Figure 3A,** the observed sensitivity to *botulinum* toxin at 39.0 °C is increased up to approximately 2 fold compared to sensitivity at 37.0 °C. Similar studies were performed using an otherwise identical cell culture meida with an osmolarity of less than 250 mOSm. The results are shown in **Figure 3B.** Sensitivity to *botulinum* toxin at 39.0 °C is increased up to approximately 7 fold compared to sensitivity to *botulinum* toxin at 37.0 °C. Surprisingly, reduced osmolarity had relatively little effect at 37 °C and actually decreased sensitivity at 35 °C, indicating a synergistic interaction between reduced osmolarity and elevated temperature.

[0048] As shown in **Figure 4A,** the sensitivity enhancing effect of elevated temperature occurs within a narrow range of temperatures. Fluorescence data from the cell-based assays performed at elevated temperatures show a loss of signal from a fluorescent protein of the construct at 41.0 °C when compared to lower temperatures, which can be indicative of poor cell health. Images of transfected cells under brightfield and fluorescence microscopy confirm poor cell health at 41.0 °C, as shown in **Figure 4B.** The transfected cells show poor morphology at 41.0°C (brightfield) and an overall decrease and diffusion of the reporter protein of the construct (YFP) at 41.0 °C.

[0049] Selectivity of the temperature effect is shown in **Figure 5.** The temperature studies shown above depict the results from using BoNT/A and transformed cells expressing a construct that can be cleaved by BoNT/A. BoNT/A and BoNT/E both cleave sites within SNAP-25, and a reporting construct incorporating SNAP-25 or a portion of SNAP-25 that includes these cleavage sites can potentially be used in the detection of either BoNT/A and BoNT/E. **Figure 5** shows the results BoNT/E cell-based assays utilizing the BOCELL™ cells described above. Surprisingly, despite utilizing the same cells and cell culture media, the use of elevated temperature within the range found to be effective for enhancement of BoNT/A assay sensitivity (i.e. 39 °C) resulted in a decrease in sensitivity for BoNT/E (shown as an elevated EC50 value). This indicates that the temperature effect may be selective for specific BoNTs.

[0050] ***Sodium Ion Effects.*** Results of studies showing the effect of reduced sodium chloride (NaCl) concentration are shown in **Figure 6A.** A custom basal cell culture media was prepared that contained no added NaCl. Variations of this custom basal media were prepared by adding NaCl at various concentrations and cell-based assays for *botulinum* toxin/A were performed using BOCELL cells. Cells were incubated for 3 hours prior to the application of media containing BoNT/A at the indicated concentrations. Fluorescence of the fluorophores (i.e. YFP and CFP) of the construct expressed by the cells was characterized 48 hours after contacting the cells with BoNT/A. The highest concentration of NaCl (48 mM) represents the NaCl content of the conventional basal cell culture media. As shown, reduction of the NaCl concentration produces a dramatic enhancement of sensitivity (indicated by reduced EC50 values), finally resulting in a nearly 50-fold increase in sensitivity in the absence of added NaCl.

[0051] The effects of reduced NaCl concentration on cell morphology (brightfield) in the absence of BoNT/A and the distribution of the construct within the transformed cells (YFP) in the absence and presence of BoNT/A after 48 hours are shown in **Figure 6B.** There is no evidence of changes in morphology or distribution of the construct at various NaCl concentrations in the cell culture media.

[0052] The impact of varying NaCl content of the cell culture media is shown in **Figure 7.** A custom basal cell culture media was prepared that contained no added NaCl. Variations of this custom basal media were prepared by adding NaCl at various concentrations and cell-based assays for *botulinum* toxin/A were performed using BOCELL cells. Basal media containing 48.3 mM NaCl represents the NaCl concentration of the conventional basal media. Cells were incubated with media containing

BoNT/A at the indicated concentrations for 48, 72, and 96 hours. Fluorescence of the fluorophores (i.e. YFP and CFP) of the construct expressed by the cells was characterized 48 hours after contacting the cells with BoNT/A. The highest concentration of NaCl (48 mM) represents the conventional NaCl content of the basal cell culture media. As shown, the concentration of NaCl has little effect on the timing of the cell-based assay.

[0053] **Figures 8A, 8B, and 8C** show typical results of studies of the effects of the timing of the introduction of low sodium content media on the sensitivity of cell-based BoNT assays. **Figure 8A** shows the results of cells carrying appropriate reporting constructs incubated in a basal media with conventional sodium content prior to exposure (i.e. pre-incubation) to a concentration of BoNT/A in low sodium content basal media for either 4 hours or 24 hours. Following these time periods the cells were transferred to basal media with conventional sodium content that contained a corresponding concentration of BoNT/A, such that the total time spent exposed to BoNT/A was 48 hours. Cells were also exposed to BoNT/A in low sodium content basal media for the entire 48 hour period to provide control conditions. **Figure 8B** shows typical results for similar studies performed using the low sodium content basal media for pre-incubation. It should be appreciated that the media used for pre-incubation of the cells had no discernible effect on the cells, indicating that preconditioning of the cells using low sodium content media is not necessary.

[0054] The effect of pre-incubation was also examined in the studies shown in **Figure 8C,** which shows typical results. Cells were pre-incubated with a supplemented conventional sodium content media (Media A) or with a low sodium content custom media (Media B). Cells were then washed briefly with either an unsupplemented conventional sodium content media (Media C) or the low sodium content custom media prior to contact with BoNT. As shown, pre-incubation in low sodium content media is not necessary to generate the enhanced BoNT sensitivity.

[0055] ***Counterion Effects.*** The sodium content of the cell culture media used in a cell-based BoNT assay of the inventive concept can be manipulated by adjusting the concentration of sodium salts other than NaCl. As shown in **Figure 9A,** a reduction in the sodium bicarbonate ($NaHCO_3$) content of a basal media is also effective at increasing the sensitivity of a cell-based BoNT assay. As shown in **Figure 9B,** just as with NaCl large improvements in sensitivity are observed over relatively small changes in sodium content.

[0056] ***Ionic Strength and Osmolarity Effects.*** The effects of removal of sodium from the media used in a cell-based BoNT assay are not due to changes in ionic strength. **Figure 10** shows typical results from studies in which a series of custom media having conventional (i.e. 70%) and reduced (i.e. 25%) sodium content and in which sodium is replaced by potassium at the same concentrations. While the enhancement of sensitivity in a cell-based BoNT assay is evident on reduction of sodium concentration, a similar enhancement is not observed when sodium is replaced with potassium and the concentration subsequently reduced. As such the effect is independent of ionic strength and can be seen as ion-specific and/or ion-selective.

[0057] **Figure 11** shows the results of supplementing low sodium content media with nonionic substances to increase ionic strength. Cell based BoNT assays were performed in culture media containing 48 mM NaCl (70% Neurobasal, total [Na+] = 53 mM), 0 mM NaCl (70% custom 0 mM NaCl, total [Na+] = 19 mM), and 0 mM NaCl media supplemented with either sucrose or trimethylamine N-oxide (TMAO). Both sucrose and TMAO are commonly used to adjust osmolarity. The sensitivity enhancement produced by the reduction of sodium in the culture media remains despite adjusting the osmolarity to the equivalent of 48 mM NaCl. The effects of reduction in sodium content in the culture media utilized in cell-based BoNT assays is therefore independent of osmolarity.

[0058] ***Mechanistic Studies of Low Sodium Content Media.*** There are a variety of mechanisms that may be involved in enhancement of the sensitivity of cell-based BoNT assays through the use of low sodium content culture media. **Figure 12A** shows typical results obtained in studies directed towards blocking cell surface receptor-mediated uptake of BoNT by cells in low sodium content culture media. Such cells were treated with a recombinant heavy chain fragment of BoNT/A (HcR/A, at 1 μM) prior to exposure of the cells to the intact BoNT/A holotoxin. Such heavy chain fragments of BoNT/A bind to the same cell surface receptors as the holotoxin but lack proteolytic activity and cannot cleave the detecting construct. As shown, blocking these receptor sites effectively blocks the toxic effects of the BoNT/A holotoxin when applied in low sodium content culture media at all but high holotoxin concentrations.

[0059] The recombinant light chain fragment of BoNT/A (Lc/A) retains the proteolytic activity of the BoNT/A holotoxin, but lacks the ability to bind to the cell surface receptors utilized for internalization of the holotoxin. **Figure 12B** shows typical results for a study on the internalization of the BoNT/A light chain by cells in low sodium content culture media. As shown Lc/A has minimal impact on these cells, indicating that nonspecific endocytosis is not a primary factor in the sensitivity enhancement seen with low sodium content media.

[0060] It should be apparent to those skilled in the art that many more modifications besides those already described are possible without departing from the inventive concepts herein. Moreover, in interpreting both the specification and the claims, all terms should be interpreted in the broadest possible manner consistent with the context. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may

be present, or utilized, or combined with other elements, components, or steps that are not expressly referenced. Where the specification claims refers to a first and a second steps, the text should be interpreted to mean that the first and second steps can be practiced in any order, not that the claim requires both element should be present or two elements are in such order. Where the specification claims refers to at least one of something selected from the group consisting of A, B, C .... and N, the text should be interpreted as requiring only one element from the group, not A plus N, or B plus N, etc. Similarly, the inventive subject matter is considered to include all possible combinations of the disclosed elements. Thus if one embodiment comprises elements A, B, and C, and a second embodiment comprises elements B and D, then the inventive subject matter is also considered to include other remaining combinations of A, B, C, or D, even if not explicitly disclosed.

Claims

1. A method of increasing the sensitivity of cell-based detection of a botulinum toxin/A (BoNT/A), comprising:

(i) providing a transfected cell that produces a construct comprising;
(a) a first terminus comprising a reporter-containing portion, wherein the reporter-containing portion exhibits a signal; and,
(b) a cleavage site that interacts with the botulinum toxin in a manner that produces a cleavage of the reporter-containing portion from a remainder of the construct;
(ii) exposing the transfected cell to the botulinum toxin at a toxin exposure temperature of from 38.5 °C to 39.5 °C, wherein sensitivity of the transfected cell's response to the Botulinum toxin at the toxin exposure temperature increases at least 2 fold compared to the transfected cell's sensitivity to the botulinum toxin at 37 °C;
(iv) obtaining the signal from the reporter-containing portion.

2. The method of claim 1 wherein the transfected cell is selected from the group consisting of a neuronal cell, a neuroendocrine tumor cell, a hybrid cell, and a stem cell.

3. The method of claim 1 wherein the reporter-containing portion comprises a first fluorophore.

4. The method of claim 1 wherein the hybrid protein further comprises a second fluorophore; preferably wherein second fluorophore is located proximal to a second terminus of the hybrid protein; or wherein the first fluorophore and the second fluorophore demonstrate ≤ 5% Forster resonance energy transfer.

5. The method of claim 1, comprising the additional step of exposing the transfected cell to a pre-toxin temperature prior to exposure to the botulinum toxin.

6. The method of claim 1 wherein the temperature of the cell prior to exposure to the botulinum toxin is between 38.5 °C and 39.5 °C.

7. The method of claim 1 wherein the sensitivity of the transfected cell's response to the botulinum toxin increases at least five fold.

8. A method of increasing the sensitivity of cell-based detection of a botulinum toxin/A (BoNT/A), comprising:

(i) providing, in a first media having a sodium concentration greater than 65 mM, a transfected cell that produces a construct comprising;

(a) a terminus comprising a reporter-containing portion, wherein the reporter-containing portion exhibits a signal; and,
(b) a cleavage site that interacts with the botulinum toxin in a manner that produces a cleavage of the reporter-containing portion from a remainder of the construct;

(ii) transferring the transfected cell to a second media having a sodium concentration of less than 50 mM;
(iii) contacting the transfected cell with the botulinum toxin; and
(iv) obtaining the signal from the reporter-containing portion.

9. The method of claim 8, wherein the second media has a sodium concentration of less than 45 mM; or wherein the second media has physiological osmotic strength.

10. The method of claim 8, wherein the sensitivity to the botulinum toxin is increased by at least a factor of 10 relative to a cell-based detection method wherein the sodium concentration of the second media is at least 65 mM.

11. The method of claim 8, wherein the transfected cell is contacted with the botulinum toxin in the second media without pre-incubation in the second media.

12. The method of claim 8, wherein the sodium concentration in the second media is reduced by a reduction in the NaCl content of a neurobasal media; or wherein the sodium concentration in the second media is reduced by a reduction in the $NaHCO_3$ content of a

neurobasal media.

Patentansprüche

1. Verfahren zum Erhöhen der Empfindlichkeit des zellbasierten Nachweises eines Botulinumtoxins/A (BoNT/A), umfassend:

   (i) Bereitstellen einer transfizierten Zelle, die ein Konstrukt erzeugt, umfassend;
   (a) einen ersten Terminus, der einen reporterhaltigen Anteil umfasst, wobei der reporterhaltige Anteil ein Signal aufweist; und,
   (b) eine Spaltstelle, die mit dem Botulinum-Neurotoxin in einer Weise interagiert, die eine Abspaltung des reporterhaltigen Anteils von einem Rest des Konstrukts bewirkt;
   (ii) Aussetzen der transfizierten Zelle dem Botulinumtoxin bei einer Toxinexpositionstemperatur von 38,5 °C bis 39,5 °C, wobei die Empfindlichkeit der Reaktion der transfizierten Zelle auf das Botulinumtoxin bei der Toxinexpositionstemperatur im Vergleich zur Empfindlichkeit der transfizierten Zelle gegenüber dem Botulinumtoxin bei 37 °C um mindestens das Zweifache zunimmt;
   (iv) Erhalten des Signals aus dem reporterhaltigen Teil.

2. Verfahren nach Anspruch 1, wobei die transfizierte Zelle ausgewählt ist aus der Gruppe bestehend aus einer neuronalen Zelle, einer neuroendokrinen Tumorzelle, einer Hybridzelle und einer Stammzelle.

3. Verfahren nach Anspruch 1, wobei der reporterhaltige Anteil einen ersten Fluorophor umfasst.

4. Verfahren nach Anspruch 1, wobei das Hybridprotein ferner einen zweiten Fluorophor umfasst, vorzugsweise
   wobei sich der zweite Fluorophor proximal zu einem zweiten Terminus des Hybridproteins befindet; oder wobei der erste Fluorophor und der zweite Fluorophor $\leq$ 5 % Forster-Resonanzenergietransfer zeigen.

5. Verfahren nach Anspruch 1, umfassend den zusätzlichen Schritt des Aussetzens der transfizierten Zelle einer Prä-Toxin-Temperatur vor der Exposition gegenüber dem Botulinumtoxin.

6. Verfahren nach Anspruch 1, wobei die Temperatur der Zelle vor der Exposition gegenüber dem Botulinumtoxin zwischen 38,5 °C und 39,5 °C liegt.

7. Verfahren nach Anspruch 1, wobei die Empfindlichkeit der Reaktion der transfizierten Zelle auf das Botulinumtoxin mindestens das Fünffache zunimmt.

8. Verfahren zum Erhöhen der Empfindlichkeit des zellbasierten Nachweises eines Botulinumtoxins/A (BoNT/A), umfassend:

   (i) Bereitstellen, in einem ersten Medium mit einer Natriumkonzentration von mehr als 65 mM, einer transfizierten Zelle, die ein Konstrukt herstellt, das Folgendes umfasst:

      (a) einen Terminus, der einen reporterhaltigen Anteil umfasst, wobei der reporterhaltige Anteil ein Signal aufweist; und,
      (b) eine Spaltstelle, die mit dem Botulinum-Neurotoxin in einer Weise interagiert, die eine Abspaltung des reporterhaltigen Anteils von einem Rest des Konstrukts bewirkt;

   (ii) Überführen der transfizierten Zelle in ein zweites Medium mit einer Natriumkonzentration von weniger als 50 mM,
   (iii) Inkontaktbringen der transfizierten Zelle mit dem Botulinumtoxin; und
   (iv) Erhalten des Signals aus dem reporterhaltigen Teil.

9. Verfahren nach Anspruch 8, wobei das zweite Medium eine Natriumkonzentration von weniger als 45 mM aufweist; oder wobei das zweite Medium eine physiologische osmotische Stärke aufweist.

10. Verfahren nach Anspruch 8, wobei die Empfindlichkeit gegenüber dem Botulinumtoxin relativ zu einem zellbasierten Nachweisverfahren um mindestens einen Faktor 10 erhöht wird, wobei die Natriumkonzentration des zweiten Mediums mindestens 65 mM beträgt.

11. Verfahren nach Anspruch 8, wobei die transfizierte Zelle in dem zweiten Medium ohne Vorinkubation in dem zweiten Medium mit dem Botulinumtoxin in Kontakt gebracht wird.

12. Verfahren nach Anspruch 8, wobei die Natriumkonzentration in dem zweiten Medium durch eine Verringerung des NaCl-Gehalts eines neurobasalen Mediums reduziert wird; oder wobei die Natriumkonzentration in dem zweiten Medium durch eine Verringerung des $NaHCO_3$-Gehalts eines neurobasalen Mediums reduziert wird.

Revendications

1. Procédé d'augmentation de la sensibilité de la détection cellulaire d'une toxine botulique/A (BoNT/A), comprenant :

(i) la fourniture d'une cellule transfectée qui produit une construction comprenant ;
(a) une première extrémité comprenant une partie contenant un rapporteur, dans lequel la partie contenant un rapporteur présente un signal ; et,
(b) un site de clivage qui interagit avec la toxine botulique d'une manière qui produit un clivage entre la partie contenant le rapporteur et le reste de la construction ;
(ii) l'exposition de la cellule transfectée à la toxine botulique à une température d'exposition à la toxine comprise entre 38,5°C et 39,5°C, la sensibilité de la cellule transfectée à la toxine botulique à la température d'exposition à la toxine augmentant d'au moins 2 fois par rapport à la sensibilité de la cellule transfectée à la toxine botulique à 37°C ;
(iv) l'obtention du signal de la partie contenant le rapporteur.

2. Procédé selon la revendication 1 dans lequel la cellule transfectée est choisie dans le groupe constitué d'une cellule neuronale, d'une cellule tumorale neuroendocrine, d'une cellule hybride et d'une cellule souche.

3. Procédé selon la revendication 1 dans lequel la partie contenant le rapporteur comprend un premier fluorophore.

4. Procédé selon la revendication 1 dans lequel la protéine hybride comprend en outre un second fluorophore ; de préférence
dans lequel le second fluorophore est situé à proximité de la seconde extrémité de la protéine hybride ; ou dans lequel le premier fluorophore et le second fluorophore présentent un transfert d'énergie par résonance de Forster $\leq$ 5 %.

5. Procédé selon la revendication 1, comprenant l'étape supplémentaire d'exposer la cellule transfectée à une température pré-toxine avant l'exposition à la toxine botulique.

6. Procédé selon la revendication 1 dans lequel la température de la cellule avant l'exposition à la toxine botulique est comprise entre 38,5°C et 39,5°C.

7. Procédé selon la revendication 1 dans lequel la sensibilité de la réponse de la cellule transfectée à la toxine botulique est multipliée au moins cinq fois.

8. Procédé d'augmentation de la sensibilité de la détection cellulaire d'une toxine botulique/A (BoNT/A), comprenant :

(i) la fourniture, dans un premier milieu ayant une concentration en sodium supérieure à 65 mM, une cellule transfectée qui produit une construction comprenant ;

(a) une extrémité comprenant une partie contenant un rapporteur, dans lequel la partie contenant un rapporteur présente un signal ; et,
(b) un site de clivage qui interagit avec la toxine botulique d'une manière qui produit un clivage entre la partie contenant le rapporteur et le reste de la construction ;

(ii) le transfert de la cellule transfectée dans un second milieu dont la concentration en sodium est inférieure à 50 mM ;
(iii) la mise en contact de la cellule transfectée avec la toxine botulique ; et
(iv) l'obtention du signal de la partie contenant le rapporteur.

9. Procédé selon la revendication 8, dans lequel le second milieu a une concentration en sodium inférieure à 45 mM ; ou dans lequel le second milieu a une force osmotique physiologique.

10. Procédé selon la revendication 8, dans lequel la sensibilité à la toxine botulique est augmentée d'au moins un facteur 10 par rapport à un procédé de détection cellulaire dans lequel la concentration en sodium du second milieu est d'au moins 65 mM.

11. Procédé selon la revendication 8, dans lequel la cellule transfectée est mise en contact avec la toxine botulique dans le second milieu sans pré-incubation dans le second milieu.

12. Procédé selon la revendication 8, dans lequel la concentration de sodium dans le second milieu est réduite par une réduction de la teneur en NaCl d'un milieu neurobasal ; ou dans lequel la concentration en sodium dans le second milieu est réduite par une réduction de la teneur en $NaHCO_3$ d'un milieu neurobasal.

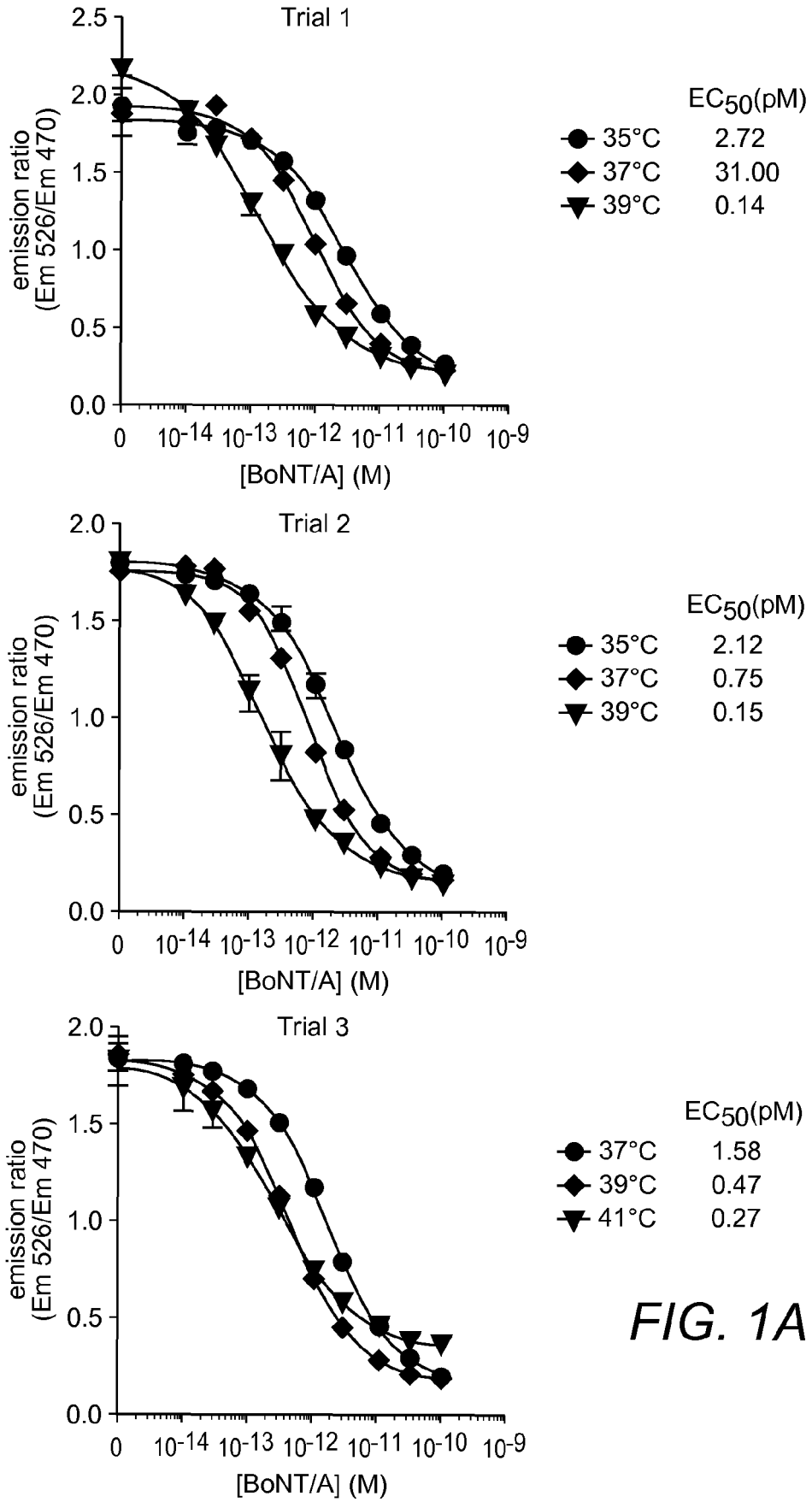
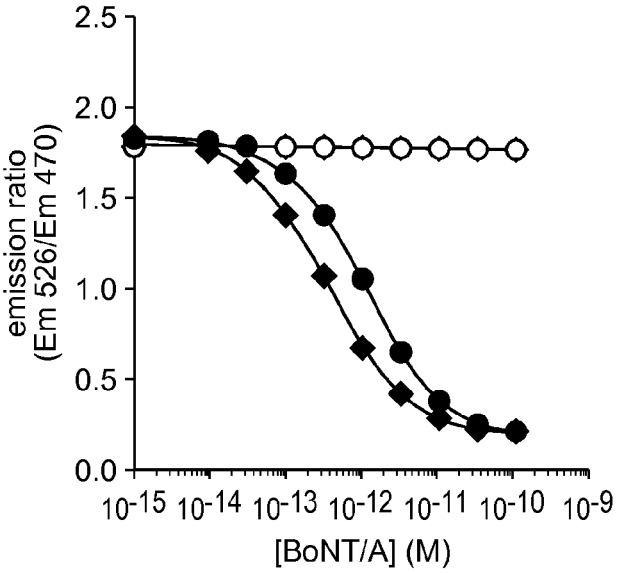
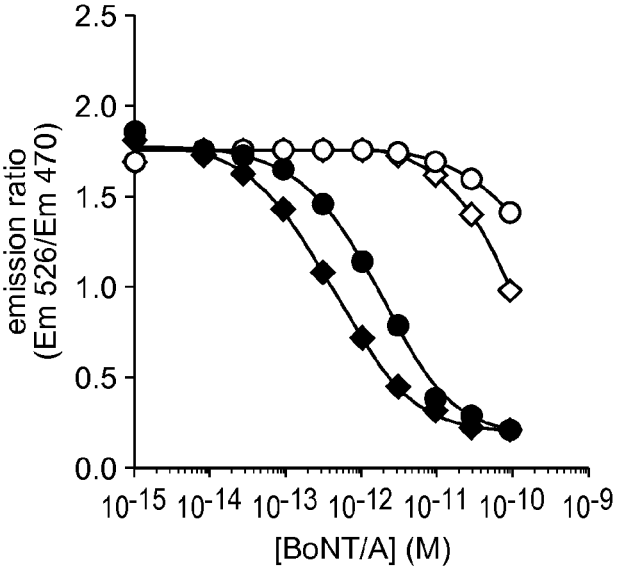

Trial 1
emission ratio (Em 526/Em 470)
2.5
2.0
1.5
1.0
0.5
0.0
0
10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)
EC50(pM)
35°C 2.72
37°C 31.00
39°C 0.14
Trial 2
emission ratio (Em 526/Em 470)
2.0
1.5
1.0
0.5
0.0
0
10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)
EC50(pM)
35°C 2.12
37°C 0.75
39°C 0.15
Trial 3
emission ratio (Em 526/Em 470)
2.0
1.5
1.0
0.5
0.0
0
10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)
EC50(pM)
37°C 1.58
39°C 0.47
41°C 0.27

FIG. 1A



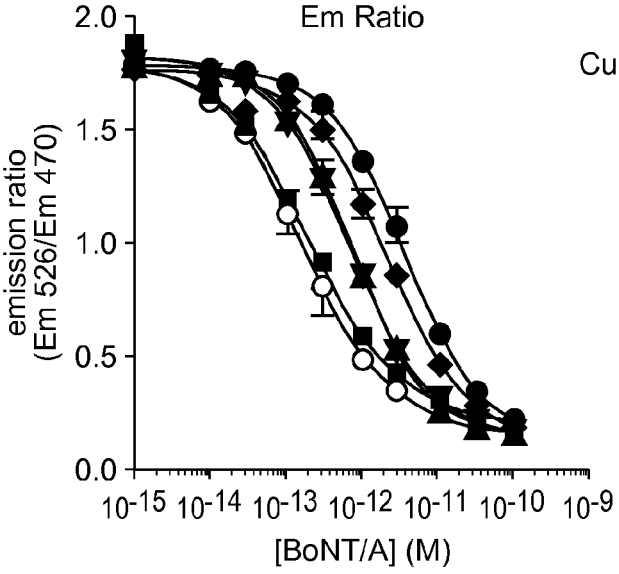
	EC_{50}(pM)
Holotoxin 37°C	1.04
Light Chain 37°C	- -
Holotoxin 39°C	0.31
Light Chain 39°C	- -

FIG. 1B



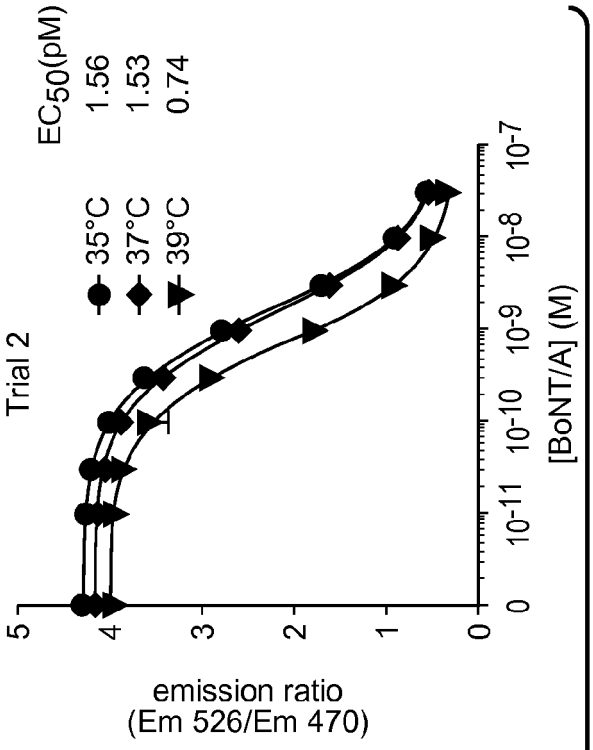
	EC_{50}(pM)
Control 37°C	1.58
HcR/A 37°C	- -
Control 39°C	0.43
HcR/A 39°C	- -

FIG. 1C



Culture/Assay Temp	EC_{50}(pM)
35/35°C	3.65
37/35°C	2.12
37/37°C	0.71
37/37°C	0.75
39/39°C	0.18
37/39°C	0.15

FIG. 2


Trial 2
EC50(pM)
35°C 1.56
37°C 1.53
39°C 0.74
emission ratio
(Em 526/Em 470)
[BoNT/A] (M)

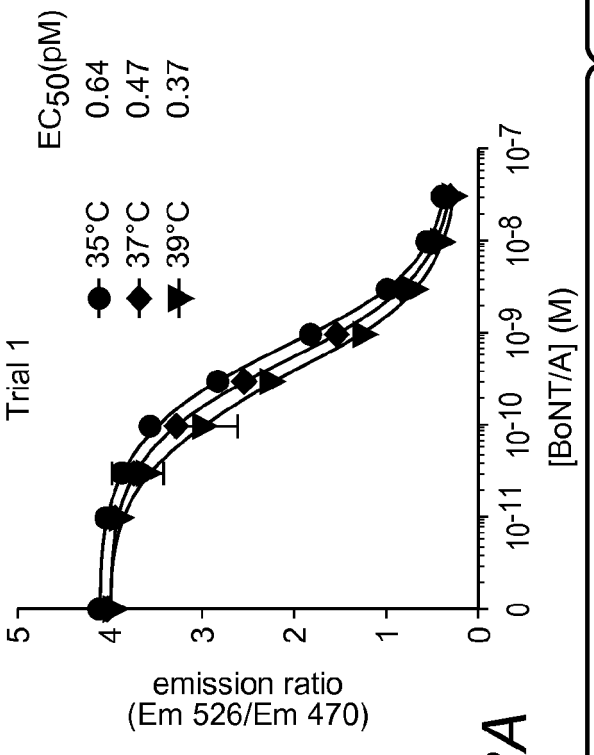
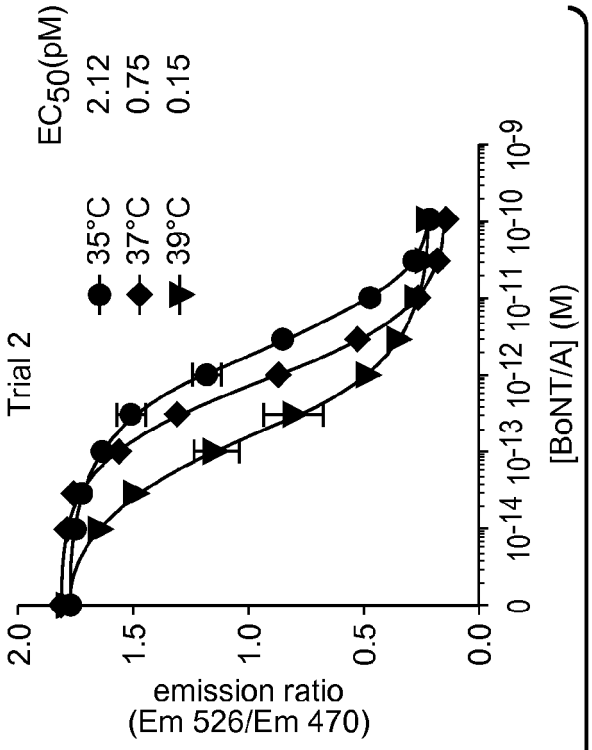

Trial 1
EC50(pM)
35°C 0.64
37°C 0.47
39°C 0.37
emission ratio
(Em 526/Em 470)
[BoNT/A] (M)

FIG. 3A


Trial 2
EC50(pM)
35°C 2.12
37°C 0.75
39°C 0.15
emission ratio
(Em 526/Em 470)
[BoNT/A] (M)

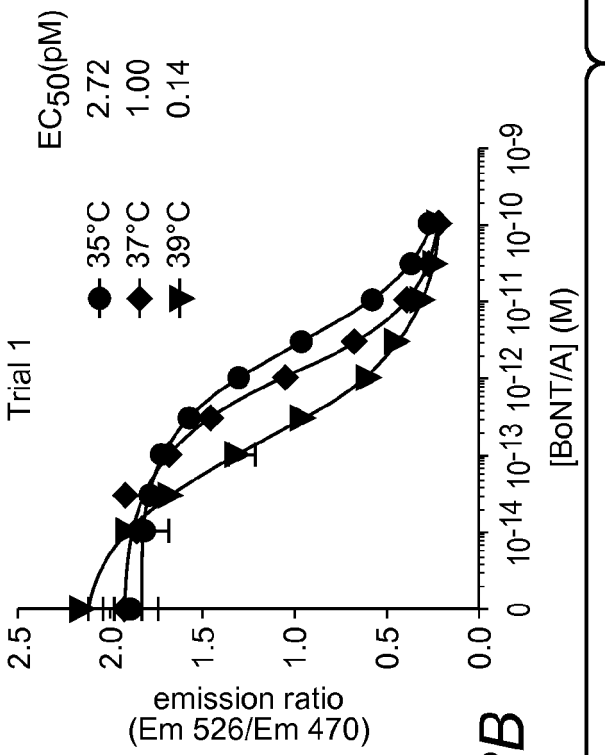
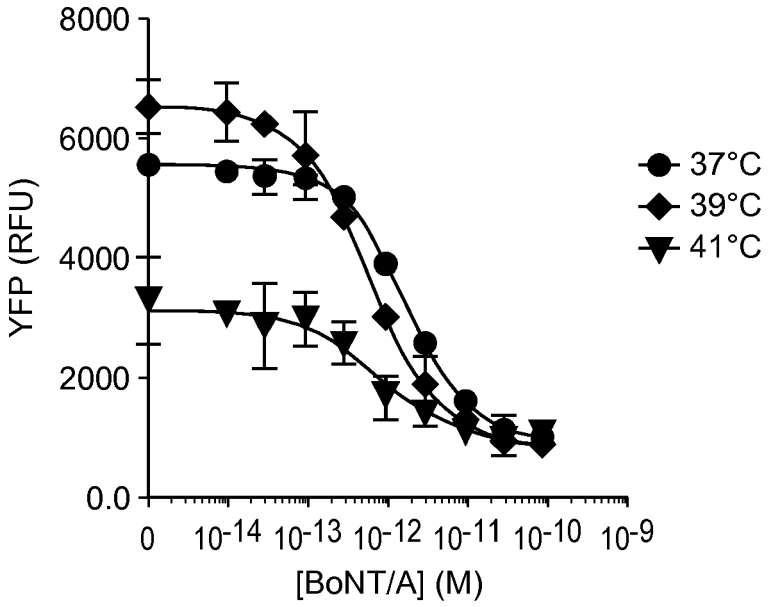

Trial 1
EC50(pM)
35°C 2.72
37°C 1.00
39°C 0.14
emission ratio
(Em 526/Em 470)
[BoNT/A] (M)

FIG. 3B

FIG. 4A


8000
6000
4000
2000
0.0
YFP (RFU)
0
10-14
10-13
10-12
10-11
10-10
10-9
[BoNT/A] (M)
37°C
39°C
41°C

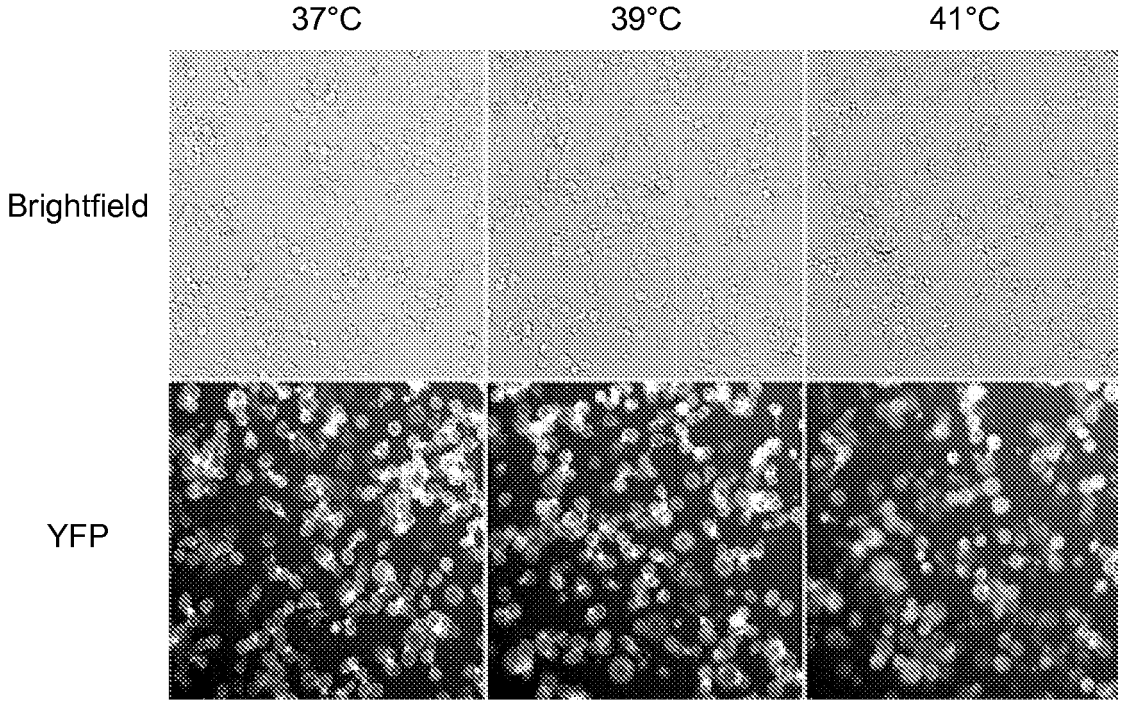
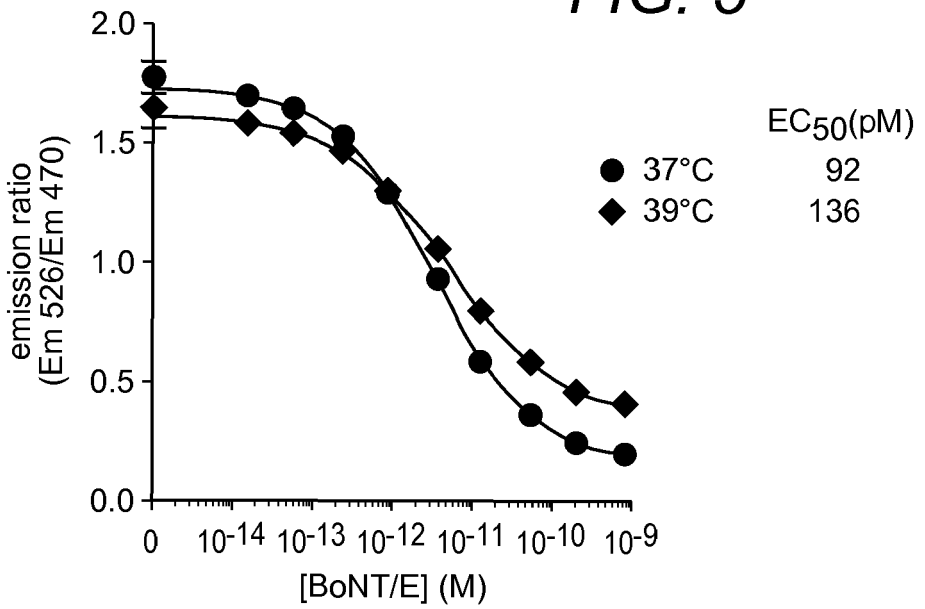

37°C
39°C
41°C
Brightfield
YFP

FIG. 4B

FIG. 5


2.0
1.5
1.0
0.5
0.0
emission ratio
(Em 526/Em 470)
0
10-14
10-13
10-12
10-11
10-10
10-9
[BoNT/E] (M)
EC50(pM)
37°C
92
39°C
136

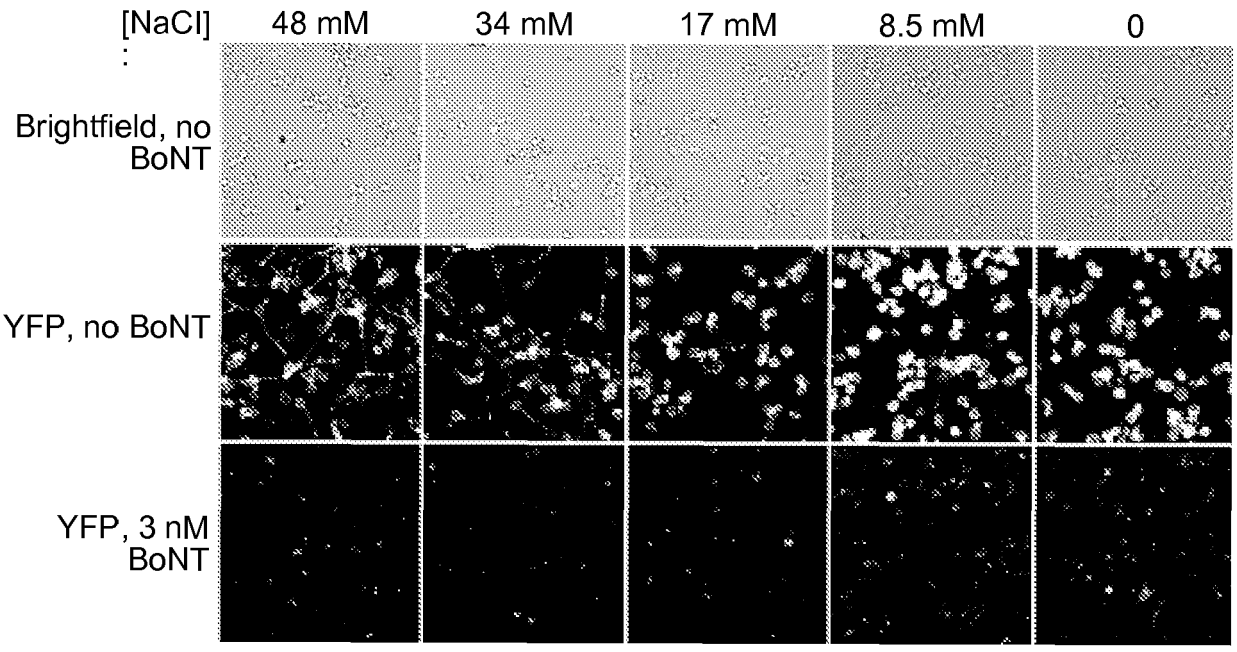

[NaCl]
48 mM
34 mM
17 mM
8.5 mM
0
Brightfield, no BoNT
YFP, no BoNT
YFP, 3 nM BoNT

FIG. 6B

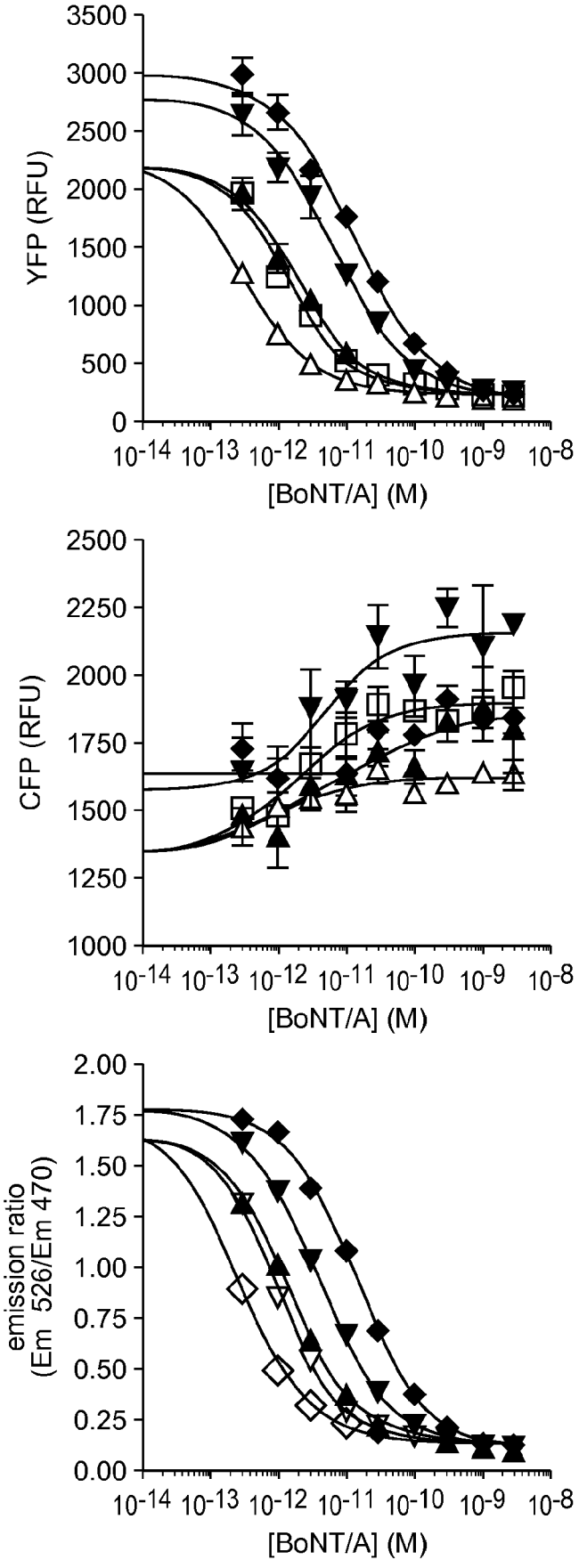


FIG. 6A

	EC_{50} (pM)
◆ 48 nM NaCl	14.1
▼ 34 nM NaCl	4.1
▲ 17 nM NaCl	1.5
▽ 8.5 nM NaCl	1.0
◇ No added NaCl	0.3

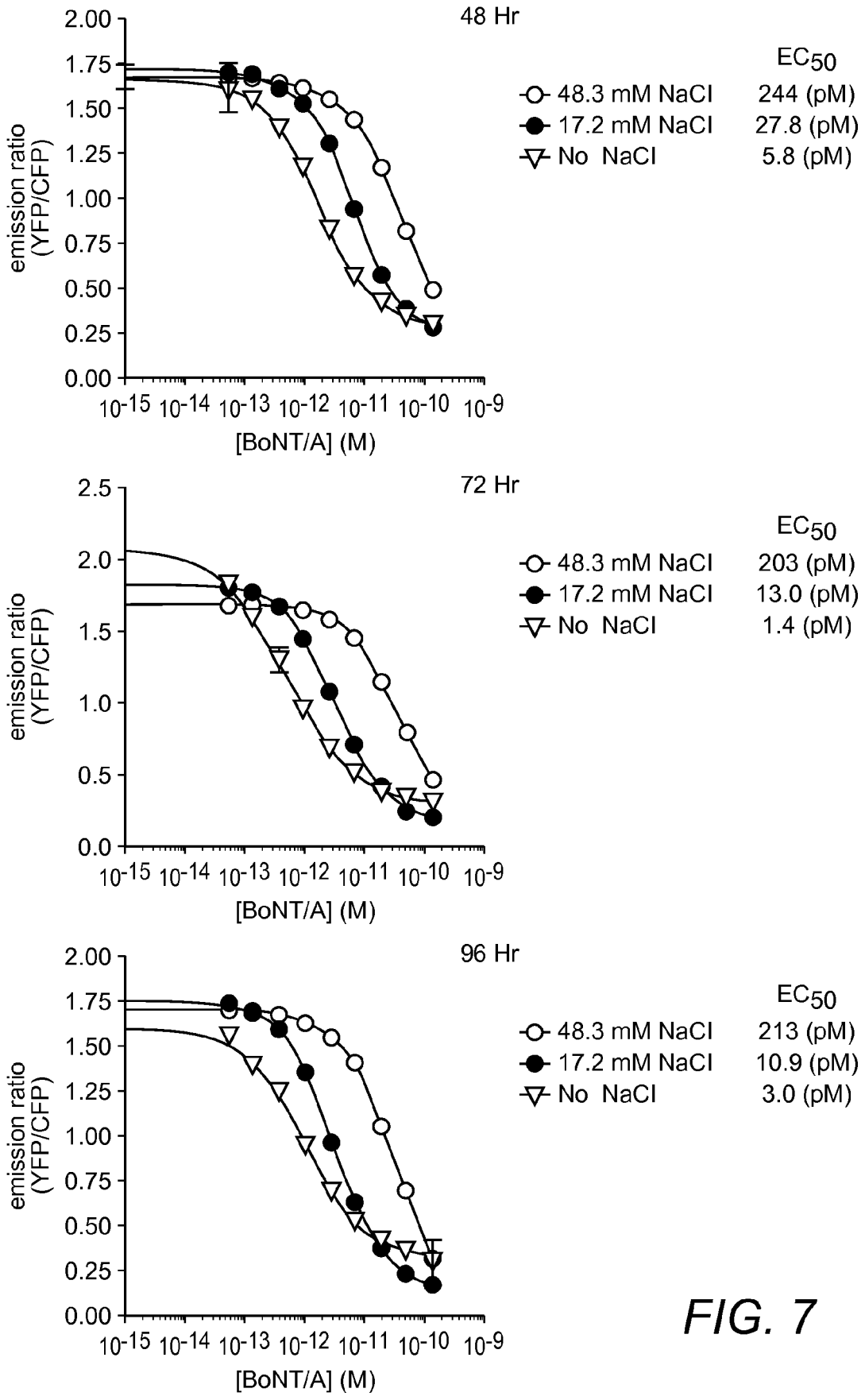

48 Hr
EC50
48.3 mM NaCl 244 (pM)
17.2 mM NaCl 27.8 (pM)
No NaCl 5.8 (pM)
emission ratio (YFP/CFP)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
10-15 10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)
72 Hr
EC50
48.3 mM NaCl 203 (pM)
17.2 mM NaCl 13.0 (pM)
No NaCl 1.4 (pM)
emission ratio (YFP/CFP)
2.5
2.0
1.5
1.0
0.5
0.0
10-15 10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)
96 Hr
EC50
48.3 mM NaCl 213 (pM)
17.2 mM NaCl 10.9 (pM)
No NaCl 3.0 (pM)
emission ratio (YFP/CFP)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
10-15 10-14 10-13 10-12 10-11 10-10 10-9
[BoNT/A] (M)

FIG. 7

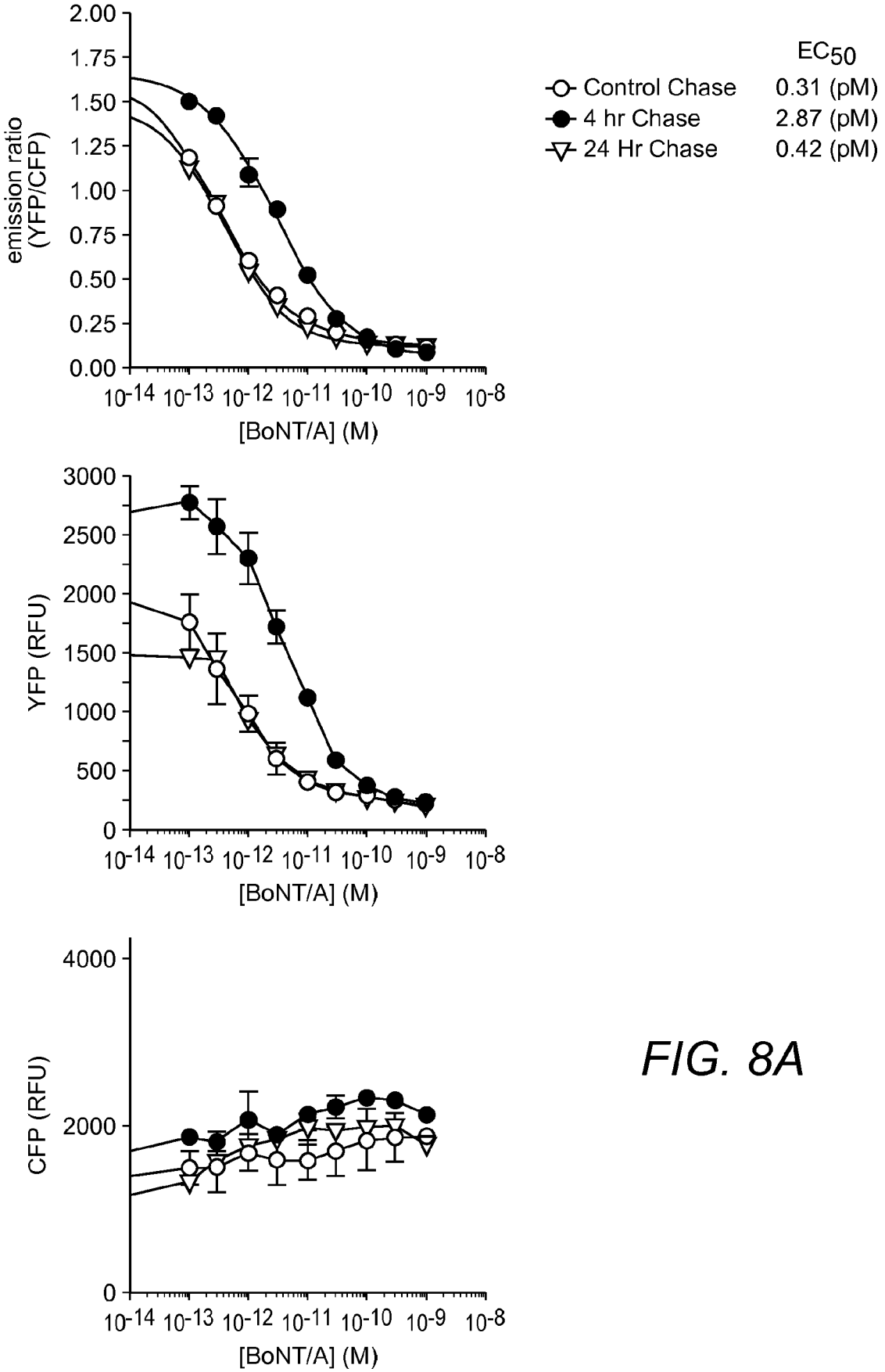

emission ratio (YFP/CFP)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
EC50
Control Chase 0.31 (pM)
4 hr Chase 2.87 (pM)
24 Hr Chase 0.42 (pM)
YFP (RFU)
3000
2500
2000
1500
1000
500
0
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
CFP (RFU)
4000
2000
0
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)

FIG. 8A

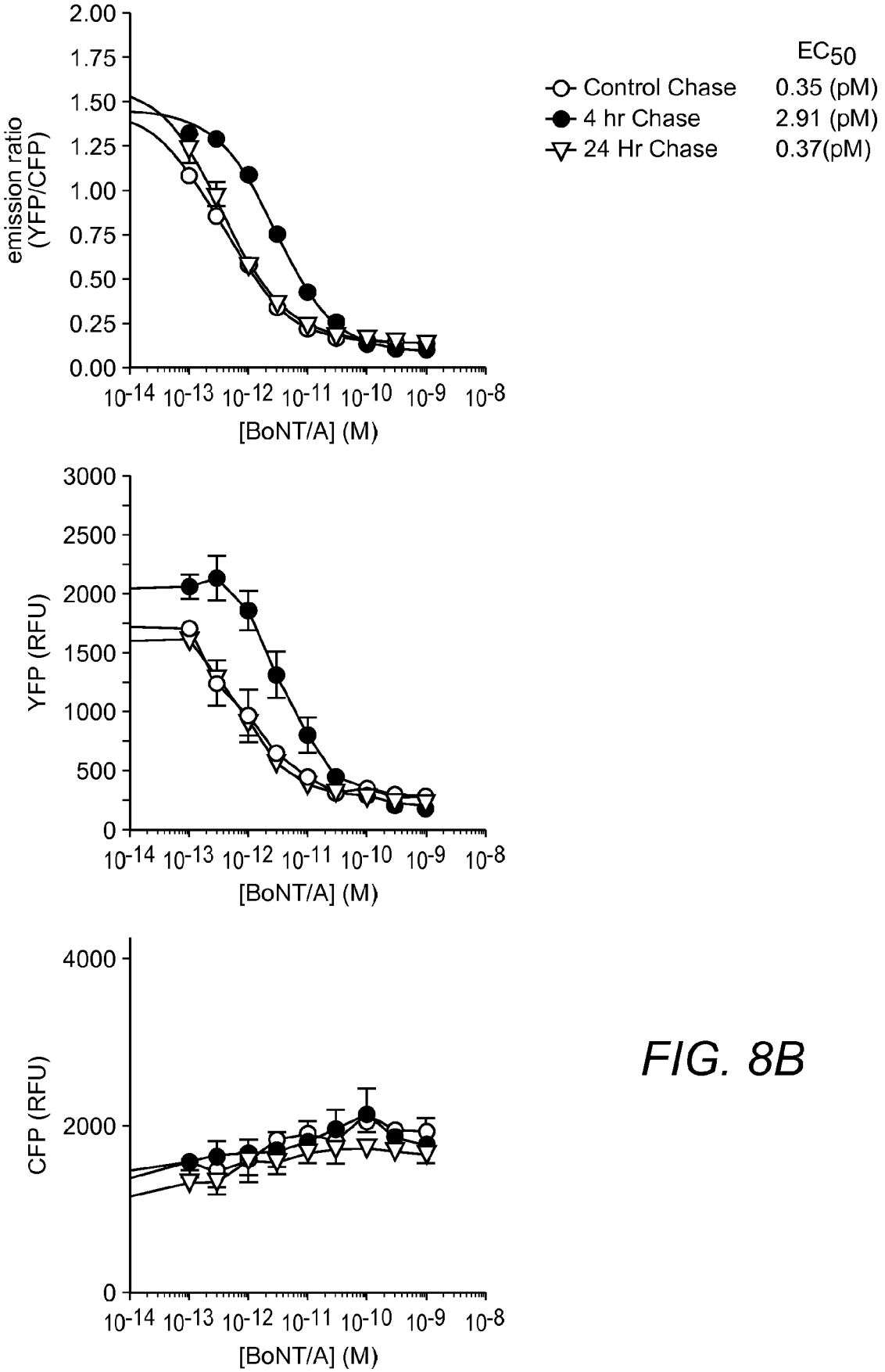

EC50
Control Chase 0.35 (pM)
4 hr Chase 2.91 (pM)
24 Hr Chase 0.37(pM)
emission ratio (YFP/CFP)
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
YFP (RFU)
3000
2500
2000
1500
1000
500
0
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
CFP (RFU)
4000
2000
0
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)

FIG. 8B

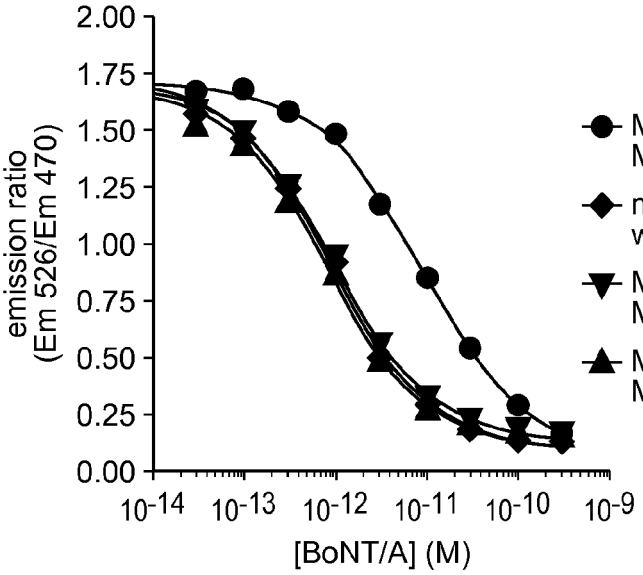


FIG. 8C

	EC_{50}(pM)
Media A preincubation, Media C wash and BoNT	9.30
media C preincubation, wash, and BoNT	0.86
Media A preincubation, Media C wash, Media B BoNT	0.94
Media A preincubation, Media B wash and BoNT	0.78

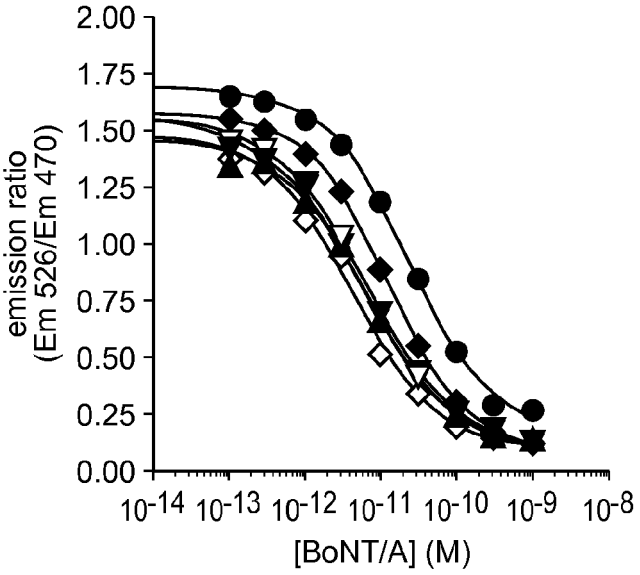


FIG. 9A

	EC_{50}(pM)
70% Neurobasal	23.7
18.3 mM NaHCO3	12.0
13.1 mM NaHCO3	5.9
6.5 mM NaHCO3	6.2
3.3 mM NaHCO3	6.1
0 mM NaHCO3	4.2

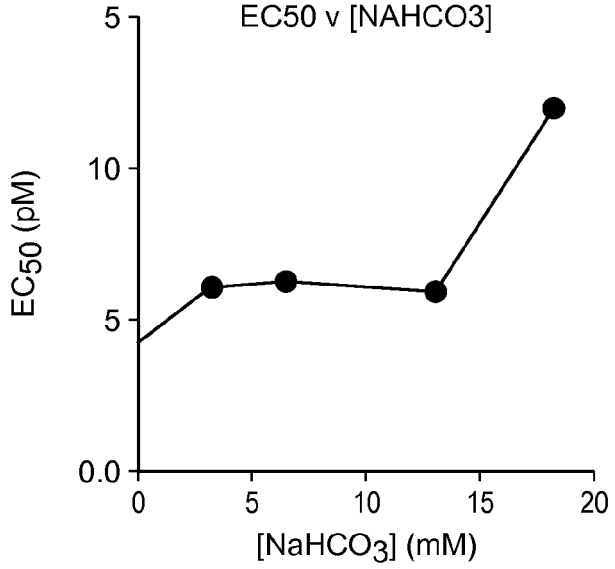


FIG. 9B

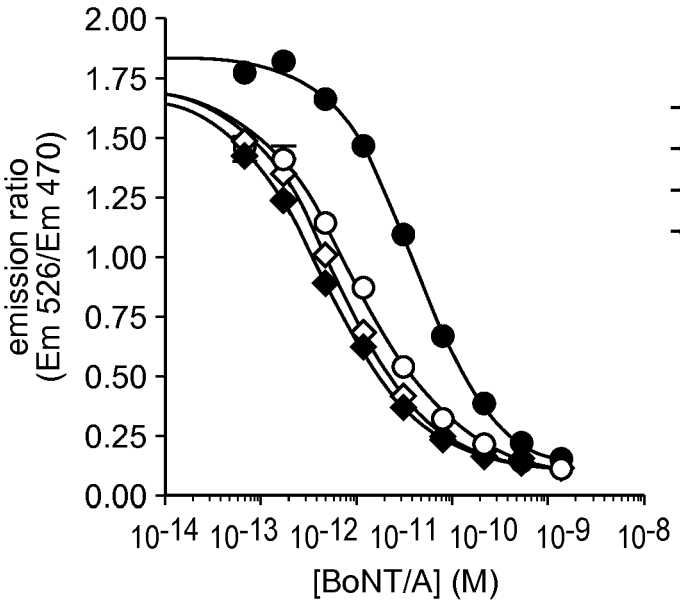

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
emission ratio
(Em 526/Em 470)
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
EC50(pM)
70% NaCl Custom 13.7
25% NaCl Custom 1.0
70% KCl Custom 2.5
25% KCl Custom 1.4

FIG. 10

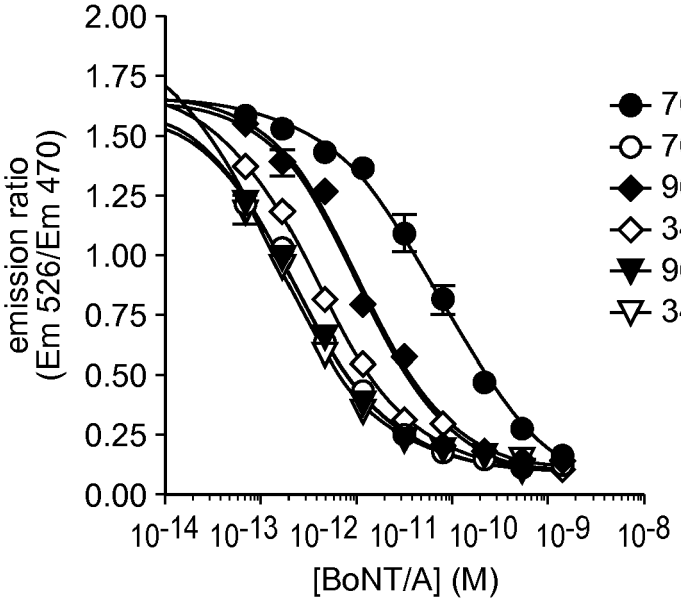

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
emission ratio
(Em 526/Em 470)
10-14 10-13 10-12 10-11 10-10 10-9 10-8
[BoNT/A] (M)
EC50(pM)
70% Neurobasal 28.4
70% Custom 0mM NaCl 0.5
96.6 mM Sucrose 2.8
34.4 mM Sucrose 0.8
96.6 mM TMAO 0.3
34.4 mM TMAO 0.3

FIG. 11

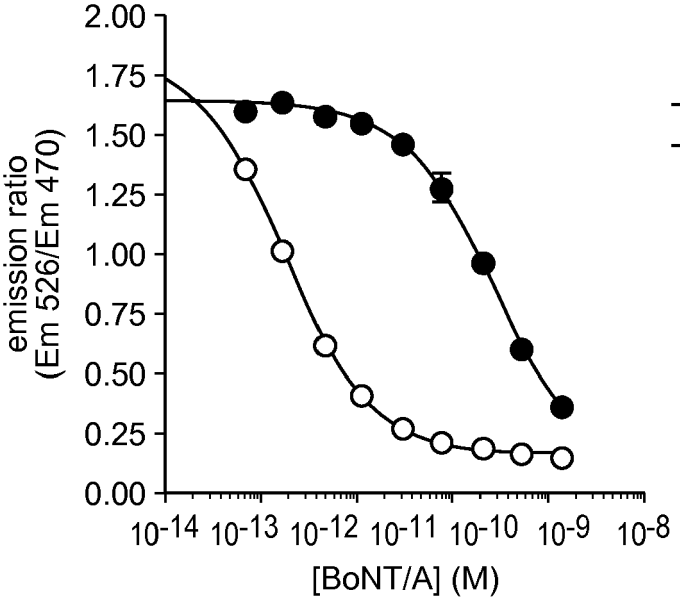


FIG. 12A

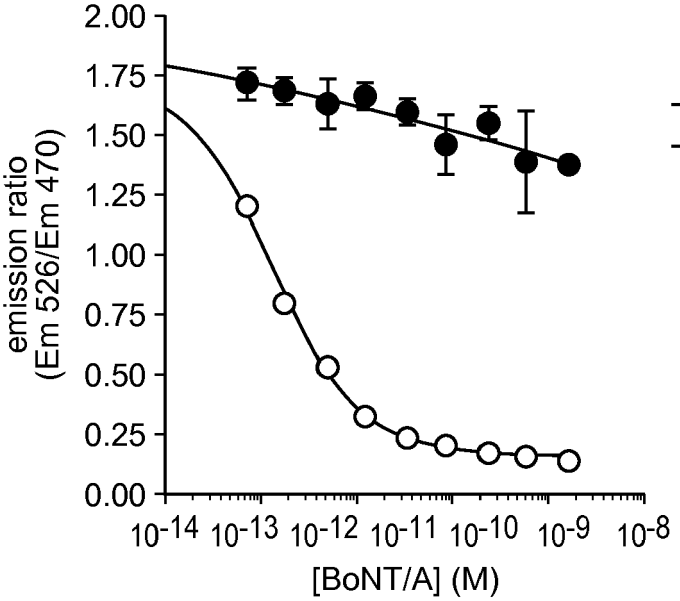


FIG. 12B

REFERENCES CITED IN THE DESCRIPTION

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- **FOSTER ; STRINGER.** Genetic Regulatory Elements Introduced Into Neural Stem and Progenitor Cell Populations. *Brain Pathology,* 1999, vol. 9, 547-567 **[0018]**