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Development of a Highly Sensitive Cell-Based Assay for Detecting Botulinum Neurotoxin Type A through Neural Culture Media Optimization

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Abstract

Botulinum neurotoxin (BoNT) is the most lethal naturally produced neurotoxin. Due to the extreme toxicity, BoNTs are implicated in bioterrorism, while the specific mechanism of action and long-lasting effect was found to be medically applicable in treating various neurological disorders. Therefore, for both public and patient safety, a highly sensitive, physiologic, and specific assay is needed. In this paper, we show a method for achieving a highly sensitive cell-based assay for BoNT/A detection using the motor neuron-like continuous cell line NG108–15. To achieve high sensitivity, we performed a media optimization study evaluating three commercially available neural supplements in combination with retinoic acid, purmorphamine, transforming growth factor β 1 (TGF β 1), and ganglioside GT1b. We found nonlinear combinatorial effects on BoNT/A detection sensitivity, achieving an EC₅₀ of $7.4 \text{ U} \pm 1.5 \text{ SD}$ (or $\sim 7.9 \text{ pM}$). The achieved detection sensitivity is comparable to that of assays that used primary and stem cell-derived neurons as well as the mouse lethality assay.

Keywords

botulinum neurotoxin; cell-based assays; media optimization; toxicology

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Declaration of Conflicting Interests

D.J.B. holds equity in Bellbrook Labs, LLC, Salus Discovery, LLC, Tasso, Inc., and Stacks to the Future, LLC. S.M.B. holds equity in Salus Discovery, LLC.

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Introduction

Botulinum neurotoxin (BoNT) is the most potent naturally produced toxin, with generally conceived lethal doses ranging from 1.3 to 13 ng/kg body weight, depending on the route of exposure,¹ and a reported lethal dose as low as 0.03 ng/kg.² Produced by *Clostridium botulinum*, there are seven designated serotypes (A–G)¹ and a hybrid of serotypes A and F³ that was first identified as novel serotype H.⁴ Despite the bioterrorism potential of BoNTs, the potency and specific biological mechanisms have found a role in medicine. Among the seven disclosed serotypes, BoNT/A is the most widely used in treating neuromuscular dystonia. Due to the extreme potency of BoNT/A, accurate quantification of the toxin is required to avoid serious side effects, including muscle numbness, difficulty breathing, and even death.⁵ Therefore, highly sensitive BoNT detection assays capable of both protecting the public against bioterrorism and ensuring the safety of patients in need of BoNT/A treatments for medical use are highly desired.

Previously, we have developed in vitro assays that leverage a microfluidic device to enable a highly sensitive measurement of the DNA content of *C. botulinum*⁶ and the catalytic activity of the toxin (i.e., proteolysis activity of light-chain catalytic domain [LC]) in food matrices such as milk and orange juice.⁷ Other laboratories also reported various in vitro biochemical detection techniques reviewed by Singh et al.^{8,9} Although these in vitro biochemical assays are suitable for infield testing against biological threats and were also shown to be highly sensitive, they do not recapitulate physiological mechanisms.

The physiological mechanism of action of BoNTs is well studied, and their structure plays an important role in imposing neurotoxicity. BoNT/A, as well as the other serotypes, weighs approximately 150 kDa, including a 50 kDa heavy-chain receptor binding domain (HCR), a 50 kDa heavy-chain translocational domain (H_N), and a 50 kDa LC. Although the HCR and H_N are covalently linked, noncovalent disulfide bonds link the LC to the heavy-chain domains. To impose toxicity, the HCR of BoNT/A associates with synaptic vesicle 2 (SV2) and ganglioside GT1b to enter cholinergic neurons upon synaptic activities.¹⁰ The endocytosed toxins are then exposed to low endosomal pH, leading to a conformational change that liberates the LC in the cytosol. The LC then cleaves SNAP25, a component of the soluble *N*-ethylmaleimide-sensitive fusion attachment protein receptor complex (SNARE), blocking neurotransmitter release and leading to the flaccid paralysis associated with botulism.^{11,12} To develop a physiologically relevant assay, each of these intoxication steps must be taken into consideration. Because the functionality and therefore lethality of BoNT cannot be determined by simple ligand-receptor binding, a system that can discern the true physiological toxicity of the toxin is required for accurate and reliable detection of BoNT.

The mouse lethality assay (MLA) is the most physiologically relevant form of the BoNT/A assay and has been considered the gold standard for detecting and determining the specific toxicity of BoNTs. In MLA, varying concentrations of BoNTs are injected into mice, and the minimal lethal dose required to kill half of the mice is denoted as 1 LD₅₀ U.¹³ Currently, the MLA has set the benchmark for detection sensitivity, achieving a sensitivity of 7–15 pg/mL (equivalent to 0.05–0.1 pM or 1–2 LD₅₀ U).¹³ Although this method has proven to be

effective, it requires special animal facilities, highly skilled technicians, and animal sacrifice, in addition to large errors ranging from 30% to 60%,¹⁴ thus, developing in vitro assays is highly desirable (reviewed by Singh et al.⁸).

Most cell-based assays involve several steps, which include culturing neuronal cells, treating the cells with BoNT/A, and directly lysing the cells to measure the amount of SNAP25 cleaved using Western blot and enzyme-linked immunoabsorbant assay (ELISA), both aided by enhanced chemiluminescence (ECL). In order for BoNT/A to enter the cells and cleave their substrates, the heavy-chain domains and LC must be intact, H_N must present LC toward cytoplasm with a conformational change occurring at endosomal pH, and finally, the LC must escape out of the endosome, reducing the disulfide bonds linked to the heavy chains.¹¹ Therefore, cell-based assay systems accurately recapitulate the physiologic intoxication mechanisms of BoNT, ensuring the full functionality and toxicity of BoNT and minimizing false readouts.

Initially, ex vivo primary spinal cord neurons demonstrated great potential, showing sensitivity comparable to that of the MLA.¹⁴ Stem cell-derived neuron-based assays were also shown to be just as sensitive.^{15–17} However, the laborious process of obtaining primary neurons still requires animal sacrifice, while differentiating stem cells takes several weeks with varying qualities of the differentiated cells.¹⁸ Despite the readily available continuous neuronal cell lines that are able to mitigate these major drawbacks of primary and stem cell-derived neurons, they have exhibited far inferior sensitivity to BoNTs.

In developing a BoNT detection assay with continuous neuronal cell lines, recent studies developed methods to improve detection sensitivities of the neuronal cell lines. The human neuroblastoma cell line SiMA exhibited an EC₅₀ (amount of BoNT/A required to cleave 50% of SNAP25) of ~6.5 pM.¹⁹ Although SiMA showed high sensitivity, the cell line is not well characterized and does not have motor neuron-like characteristics. BoNTs enter neurons at the neuromuscular junction, which is formed by the synaptic terminal of motor neurons and skeletal muscle fibers. Thus, scouting and using cell lines with motor neuron-like characteristics will offer the most representative physiologic response. NG108–15 is a cholinergic neuronal cell line that was shown to form functional synapsis with skeletal muscle fibers^{20,21} and extend neurites when cultured with Schwann cells differentiated from adipose-derived stem cells,²² indicating motor neuron-like characteristics. However, NG108–15 cells suffer from low BoNT/A detection sensitivity in their native state. Previously, we observed enhanced BoNT/A detection sensitivity when cocultured with Schwann cells, which demonstrated the potential for sensitizing and further strengthening motor neuron characteristics of the NG108–15 cell line.²³ Additionally, Whitemarsh et al. showed that culturing the NG108–15 cell line in a neural differentiation medium increased the BoNT/A detection sensitivity to an EC₅₀ of ~11.5 U (equivalent to ~12.3 pM) upon addition of GT1b a day prior to treating cells with BoNT/A, but the effects of the different components were not thoroughly investigated.²⁴ Despite these advances, further interrogation of NG108–15 cells and their culture conditions could provide the advanced sensitivity needed to allow their use in an in vitro cell-based BoNT detection assay.

In this study, we focused our efforts on optimizing neural culture media to further assess the effects of different media components by improving neural cell culture conditions with the continual neuron cell line NG108–15. We screened and looked at the combinatorial effect of three commercially available neural supplements. Additionally, varying concentrations of retinoic acid (RA), purmorphamine (Pur), transforming growth factor β 1 (TGF β 1), and GT1b were tested for their impact. Through our optimization study, we developed a highly sensitive cell-based assay that can detect BoNT/A without introducing media additives throughout the culture, and achieved higher sensitivity than the previous report that used NG108–15.

Materials and Methods

Cell Culture

NG108–15 cells were purchased from ATCC (Manassas, VA) and were maintained as suggested by the supplier. Briefly, Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) basal medium that was supplemented with hypoxanthine, aminopterin, and thymidine (HAT supplement; Sigma, St. Louis, MO), 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin (P/S; Gibco, Carlsbad, CA) was used to expand and maintain the cell line. To initiate the BoNT/A detection assay, NG108–15 cells were plated at ~15,000 cells/well in a 96-well plate coated with 1:100 diluted growth factor reduced Matrigel (BD Biosciences, San Jose, CA). The cells were seeded in full growth medium and incubated at 37 °C with 5% CO₂ overnight. On the following day, the complete growth medium was replaced with neural differentiation media. The neural differentiation media contained varying combinations of neural supplements B27, N2 (Invitrogen), and GS21 (GlobalStem, Rockville, MD) in Neurobasal medium containing GlutaMAX (Invitrogen) and P/S. The neural differentiation media was then further supplemented with varying concentrations of retinoic acid (Sigma), purmorphamine (Cayman Chemical, Ann Arbor, MI), GT1b (Matreya, Pleasant Gap, PA), and TGF β 1 (PeproTech, Rocky Hill, NJ). The neural differentiation media was replaced every other day for 6 days. On day 7, varying concentrations of BoNT/A (8 pg/U specific toxicity; Johnson Laboratory, University of Wisconsin in Madison, WI) were diluted in 50 μ L of the respective differentiation media and incubated for an additional 2 days. Cells were then directly lysed using lithium dodecyl sulfate (LDS) buffer (Invitrogen) prepared with a protease inhibitor cocktail (Thermo Scientific, Rockford, IL) before being collected for Western blot analysis.

Western Blot

Gel electrophoresis was performed using Novex NuPAGE 12% Bis-Tris gel in sodium dodecyl sulfate (SDS) MOPS running buffer (Invitrogen) at 200 V for 1 h. The gel was then transferred onto a nitrocellulose membrane using the TransBlot Turbo Transfer System (Bio-Rad, Hercules, CA), blocked with 5% nonfat dried milk, and stained with primary and subsequently secondary antibodies. Mouse anti-SNAP25 at 1:4000 dilution (Synaptic Systems, Gottingen, Germany) and mouse anti- β -actin at 1:10,000 dilution (Sigma) were used for the primary antibodies. Anti-mouse IR800 (Li-Cor) at 1:10,000 dilution was used as the secondary antibody. An Odyssey Infrared Imager (Li-Cor) was used to scan blots, and

the relative amount of SNAP25 cleavage was quantified using ImageJ (National Institutes of Health, Bethesda, MD).

Statistical Analysis

Prism GraphPad 6.0 (La Jolla, CA) was used to fit dose–response curves, computing the EC_{50} of the SNAP25 cleavage, and for all statistical analysis. Error bars on all dose–response curves and EC_{50} bar graphs indicate standard errors. All experiments were repeated at least three times.

Safety Considerations

BoNT/A is highly toxic and must be handled carefully in a biosafety hood. All materials and supplies that were in contact with BoNT/A were disinfected using 10% bleach for a minimum of 10 min before being discarded in biohazard waste containers.

Results and Discussion

Neural Culture Media Supplements

We began our media optimization studies by confirming the previously reported EC_{50} of ~60 U (measured by amount of BoNT/A required to cleave 50% of SNAP25) upon differentiating NG108–15 in neural differentiation medium supplemented with B27.²⁴ After differentiating NG108–15 cells for 6 days, followed by 2 days of BoNT/A treatment, we obtained a similar EC_{50} of $67.2 \text{ U} \pm 18.0$ standard deviation (SD) in B27-supplemented Neurobasal media (Fig. 1A,B). Additionally, we tested two more neural supplements, N2 and GS21, to generate baselines on the effects of the three neural supplements on BoNT/A detection sensitivity in NG108–15. Interestingly, culturing NG108–15 in GS21 neural supplement significantly reduced the EC_{50} to $27.4 \text{ U} \pm 5.5$ SD, whereas the cells in N2 neural-supplemented media failed to show a statistical significance, with an EC_{50} of $99.5 \text{ U} \pm 8.2$ SD against B27 (Fig. 1A,B). This experiment showed that the neural supplement alone significantly affects BoNT/A detection sensitivity, as GS21 appeared superior to the other tested supplements in facilitating BoNT/A detection.

B27 is the most widely used neural culture supplement. However, its formulation is kept proprietary and the lot-to-lot variability has been a concern.²⁵ In the efforts to reduce the lot-to-lot variability and fully define the elements required to culture neural cells, Chen et al. developed a new neural supplement (NS21) that contains 21 components (GS21 is a commercially available analogue of NS21) and disclosed its full formulation. NS21 was shown to be a superior alternative to B27 in hippocampal neuron culture that reduced the degeneration of soma and axons that were seen in B27-supplemented media.²⁶ Also evident in our results, GS21 provided a different environment for neuronal cell culture, and culturing NG108–15 in GS21-supplemented medium significantly improved the condition for BoNT/A detection. The increased sensitivity with GS21 neural supplement was encouraging, and we moved forward to media optimization.

Concentrated Neural Supplements in Combination with RA and Pur

N2 neural supplement contains five components that can be used minimally for culturing neuronal cells compared to the B27 and GS21 we tested. Although these neural supplements are preformulated to the recommended final overall concentration at 1×, using them at higher concentrations and in combinations were limited to B27 and N2 at 1× concentration.¹⁹ We hypothesized that the quantity of specific components in each neural supplement may be limiting; thus, increasing the concentrations or using them in combination may enhance the neural cell culture condition and increase BoNT/A detection sensitivity.

To begin with, we designed a five-factor four-level Taguchi design of experiments (Suppl. Table S1) that takes account of the varying concentrations of neural supplements in combination with RA and Pur. RA and Pur were tested because they were shown to increase BoNT/A detection sensitivity²⁴ and are required to differentiate human pluripotent stem cell–derived neural progenitor cells toward the motor neuron lineage.²⁷ The measured EC₅₀ was the lowest at 2× B27, 3× N2, and 4× GS21 in combination with 2.5 μM Pur, followed by 2× B27, 4× N2, and 3× GS21 (Suppl. Fig. S1A). Next, we analyzed the main effect of the means and signal-to-noise ratios to identify suggested optimal concentrations of the five factors that were tested. The analysis indicated that adding 2× B27, 4× N2, and 3× GS21 to 2.5–10 μM of RA and 2.5 or 10 μM of Pur is the most probable optimized culture condition (Suppl. Fig. S1B,C).

After analyzing Taguchi's design of experiments, we narrowed the study parameters for further investigation. The concentrations of the neural supplements were fixed based on the EC₅₀ in Supplemental Figure S1A and the main effect and signal-to-noise plots in Supplemental Figure S1B,C to either 2×, 3×, and 4× or 2×, 4×, and 3× concentrations of B27, N2, and GS21, respectively. Tested concentrations of RA and Pur ranged from 2.5 to 10 μM. As baselines, a 1× concentration of each neural supplement and all three combined at 1× each were also tested (Suppl. Table S2). Surprisingly, only the presence and the absence of GS21 in media showed statistical significance. The results also suggested that the differences in the EC₅₀ that we obtained from the Taguchi study were mainly due to the addition of RA and Pur, rather than the concentrations of neural supplements. It appeared that higher concentrations of neural supplements in the culture media did not significantly contribute to BoNT/A detection sensitivity (Fig. 2). It is also interesting to note that the EC₅₀ diverged with the increased amount of RA in different combinations of neural supplements (study conditions 9–12 vs. 18–21 in Suppl. Table S2). When computed using the Student two-tailed *t* test, the conditions containing different combinations of neural supplements but the same amount of RA and Pur (study conditions 12 and 21 in Suppl. Table S2) showed a significant difference in the EC₅₀ (Fig. 2), confirming that all the parameters we tested so far have a nonlinear relationship.

Combination of Neural Supplements

The previous set of experiments showed that the components in at least GS21 are not limiting. Additionally, it was surprising that GS21 was the only significant contributor to decreasing the EC₅₀ in BoNT/A detection sensitivity (Fig. 2). To further determine the optimized media condition for culturing neurons to increase BoNT/A detection sensitivity,

we lowered the concentrations of neural supplements to 1× and tested the following conditions: (1) GS21 alone, (2) GS21 and N2, and (3) GS21, N2, and B27. In this study, we saw a significant decrease in the EC₅₀ only when GS21 was combined with N2, which also indicated that components in these two neural supplements acted synergistically in the detection sensitivity. Although adding B27 to GS21 and N2 combined media also lowered the EC₅₀, it failed to show statistical significance, thus suggesting a possible negative effect (Fig. 3A,B).

Combinatorial Effect of Neural Supplements, RA, and Pur

Finally, we deduced the desired amount and combination of neural supplements. Yet, the concentrations of RA and Pur required to achieve the optimal medium for the highest sensitivity were not evaluated. In this set of experiments, we decided to fix the concentration of Pur at 7.5 μM instead of testing all combinations of RA and Pur. The concentration of Pur was determined by the observation where we saw a diverging trend of the EC₅₀ with the addition of RA (Fig. 2). Additionally, we used 2.5 and 10 μM RA (the lowest and the highest concentration we tested throughout this report) to test whether RA works with or against the neural supplements and Pur. Although GS21 combined with N2 showed the lowest EC₅₀, we evaluated all three combinations of neural supplements that were tested in Figure 3 in case of possible nonlinear combinatorial effects of RA and Pur.

In conditions that only contained neural supplement GS21 (group 1), the lowest EC₅₀ was achieved when 2.5 μM RA and 7.5 μM Pur were added. Increasing the concentration of RA from 2.5 to 10 μM did not show a significant change in the EC₅₀ (Fig. 4A,D). In media supplemented with GS21 and N2 (group 2), we did not see any statistical difference when RA and Pur were introduced. However, in contrast to group 1, increasing RA from 2.5 to 10 μM significantly increased EC₅₀ (Fig. 4B,D). Lastly, a significant decrease in the EC₅₀ was observed with the addition of 2.5 μM RA in the group that contained all three neural supplements (group 3), and it was the most BoNT/A-sensitive condition of all (Fig. 4C,D).

From the results of Figure 3, we expected the most sensitive condition to contain only GS21 and N2 neural supplements in addition to RA and Pur. To our surprise, we saw the most gain in sensitivity in the media that contained all three supplements. When we performed a group *t* test of the means, groups 1–3 and groups 2 and 3 were statistically significant (Fig. 4). This set of experiments again indicated a nonlinear combinatorial effect of the components contributing to increased BoNT/A detection sensitivity.

Effect of TGFβ1 and GT1B

After thorough investigation on the effect of the neural supplements RA and Pur on BoNT/A detection sensitivity, we tested two more media additives that might further enhance our assay. In a previous study, we showed a 16-fold increase in the BoNT/A detection sensitivity when NG108–15 cells were cocultured with Schwann cells.²³ Schwann cells were shown to promote synaptogenesis and synaptic activities of the primary spinal cord motor neurons by secreting TGFβ1.^{28,29} In addition, ganglioside GT1b was shown to significantly increase the BoNT assay detection sensitivity.^{19,24} Therefore, we investigated the effect of GT1b and

TGFβ1 in our final media that contained all three neural supplements, 2.5 μM RA, and 7.5 μM Pur.

At 50 ng/mL of TGFβ1, the EC₅₀ was 7.4 U ± 1.5 SD without a statistical significance against the control (Fig. 5A,B). Despite previous reports that show GT1b increases the BoNT/A detection sensitivity, we did not see additional benefits of adding GT1b to our culture medium (Fig. 5C,D). Furthermore, the addition of both TGFβ1 and GT1b did not show any gain in the detection sensitivity (Fig. 5E,F). The final medium that included TGFβ1 reached the limit of detection between 0.3 and 1.2 U (Fig. 6).

We focused our efforts on increasing the sensitivity of the motor neuron-like NG108–15 cell line through combinatorial optimization of the neural cell culture medium with the final formulation of 1× B27, 1× N2, 1× GS21, 2.5 μM RA, 7.5 μM Pur, and 50 ng/mL TGFβ1 to achieve a detection sensitivity of EC₅₀ 7.4 U ± 1.5 SD (equivalent to ~7.9 pM), with a detection limit between 0.3 and 1.2 U. The sensitivity does not surpass that of in vivo MLA (1–2 LD₅₀ U) and ex vivo primary (EC₅₀ 3.8 U) and in vitro stem cell-derived neuron (EC₅₀ 0.98 U) cell-based assays,^{14,16} but the detection range is comparable.

The cell-based assay we report here is suitable for testing pharmaceutical reagents to determine the concentration and activity of the toxin and is not intended for use as the primary assay for infield testing. The assay, however, can be accompanied with biochemical detection assays that are amenable for infield testing to accurately determine the toxicity if BoNT is detected in food supplies. We quantified the relative amount of SNAP25 cleavage using Western blot as the end-point measurement to assess the specificity of BoNT/A and the sensitivity of the assay. Although Western blot is not amenable for high throughput measurements and is difficult to validate, it can be replaced with ELISA, as Fernandez et al. demonstrated with a proprietary antibody specific to cleaved SNAP25. The ELISA method for detection also attributed to a twofold gain in the sensitivity,¹⁹ which may be applicable to our culture to further improve our method.

Through step-by-step media optimization study, we developed a highly sensitive cell-based BoNT/A detection assay using a neuronal cell line that was characterized to behave like motor neurons. The assay sensitivity is comparable to the sensitivity reported with the MLA and eliminates the need for the animal sacrifice and laborious steps involved in obtaining primary or stem cell-derived neurons. This approach provides a good example of the development and refinement of new in vitro cell-based approaches for BoNT detection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

SNAP25 cleavage in different neural supplements. **(A)** Dose–response curve of SNAP25 cleavage in NG108–15 cells cultured in Neurobasal medium supplemented with B27 (EC₅₀ 75.0 U ± 18.0 SD), N2 (EC₅₀ 99.5 U ± 8.2 SD), or GS21 (EC₅₀ 27.4 U ± 5.4 SD). **(B)** The dose–response curves were analyzed to calculate the amount of BoNT/A required to cleave 50% of SNAP25, denoted EC₅₀, and plotted. One-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test was used for statistical analysis (**, $p < 0.005$; ***, $p < 0.001$).

Figure 2.

Effect of RA and Pur in concentrated neural supplement media. The EC₅₀ of SNAP25 cleavage was plotted in NG108–15 cells cultured in Neurobasal media supplemented with 1× concentrations of B27, N2, and GS21, or all three supplements were added at 1× concentrations each of B27, N2, and GS21 combined (black). A significant decrease in the EC₅₀ was observed in media containing GS21 and all three supplements compared to media with B27 or N2 alone. Increased concentrations of the neural supplements at 2× B27, 3× N2, and 4× GS21 (red) and 2× B27, 4× N2, and 3× GS21 (blue) were tested with varying concentrations of RA and Pur. All EC₅₀ and SD values are tabulated in Supplemental Table S3. No significance was observed compared to GS21 alone or combined B27, N2, and GS21 at a 1× concentration each. One-way ANOVA followed by Tukey's multiple comparison test was used for statistical analysis (****, $p < 0.0001$). The Student t test was used to compare the statistical differences between the conditions containing different neural supplements but with same amount of RA and Pur (###, $p < 0.001$).

Figure 3.

Effect of GS21 in combination with N2 and B27 neural supplements. **(A)** Dose–response curve of SNAP25 cleavage in different combinations of neural supplement each at a $1 \times$ concentration. **(B)** The analyzed EC₅₀ of the three tested conditions ($27.4 \text{ U} \pm 5.4 \text{ SD}$, $17.7 \text{ U} \pm 2.7 \text{ SD}$, and $19.1 \text{ U} \pm 5.8 \text{ SD}$ for GS21; GS21 and N2; and GS21, N2, and B27, respectively) showed a significant decrease in the EC₅₀ when GS21 was supplemented with N2. One-way ANOVA followed by Tukey’s multiple comparison test was used for statistical analysis (*, $p < 0.05$).

Figure 4.

Combinatorial effects of RA and Pur in media supplemented with GS21, N2, and B27. (A) Dose–response curve of SNAP25 cleavage when cultured in media supplemented with GS21 only, (B) GS21 and N2 combined, and (C) GS21, N2, and B27 combined. To all neural supplement media, varying concentrations of RA and Pur were added to test their combinatorial effects on the EC₅₀. The colors of both the dose–response curves and EC₅₀ plots indicate the amount of RA and Pur added to the media: no RA and no Pur (black), 2.5 μM RA and 7.5 μM Pur (red), and 10 μM RA and 7.5 μM Pur (blue). (D) Analyzed EC₅₀ values of the dose–response curves were plotted. All EC₅₀ and SD values are tabulated in Supplemental Table S3. One-way ANOVA followed by Tukey’s multiple comparison test was used for statistical analysis, and the *p* value against the condition that contained only GS21 (group 1, black bar) is marked with an asterisk, and the rest are indicated with a pound sign. A multiple-group *t* test of the means ($\ddagger$) showed statistical significance of group 3 to both group 1 and group 2. For all statistical tests, *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.0001; #, *p* < 0.05; ####, *p* < 0.0001; and $\ddagger$, *p* < 0.05.

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Figure 6.

Western blot of SNAP25 cleavage in the final medium. The Western blot image stained with SNAP25 antibody shows cleaved and uncleaved portions of the SNAP25 protein in the final medium composed of 1× B27, 1× N2, and 1× GS21 neural supplements and 2.5 μ M RA, 7.5 μ M Pur, and 50 ng/mL TGF β 1 additives. The lower bands indicate cleaved SNAP25 and visible starting at 0.3 U.