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Use of *Brevibacillus choshinensis* for the production of biologically active brain-derived neurotrophic factor (BDNF)

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Abstract Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family critical for neuronal cell survival and differentiation, with therapeutic potential for the treatment of neurological disorders and spinal cord injuries. The production of recombinant, bioactive BDNF is not practical in most traditional microbial expression systems because of the inability of the host to correctly form the characteristic cystine-knot fold of BDNF. Here, we investigated *Brevibacillus choshinensis* as a suitable expression host for bioactive BDNF expression, evaluating the effects of medium type (2SY and TM), temperature (25 and 30 °C), and culture time (48–120 h). Maximal BDNF bioactivity (per unit mass) was observed in cultures grown in 2SY medium at extended times (96 h at 30 °C or >72 h at 25 °C), with resulting bioactivity comparable to that of a commercially available BDNF. For cultures grown in 2SY medium at 25 °C for 72 h, the condition that led to the greatest quantity of biologically active protein in the shortest culture time, we recovered 264 μg/L of BDNF. As with other microbial expression systems, BDNF aggregates did form in all culture conditions, indicating that while we were able to recover biologically active BDNF, further optimization of the expression system could yield still greater quantities of bioactive protein. This study provides confirmation that *B. choshinensis* is capable of producing biologically active BDNF and that further optimization of culture conditions could prove valuable in increasing BDNF yields.

Keywords BDNF · Brain-derived neurotrophic factor · *Brevibacillus choshinensis* · Bioactive · Cystine-knot protein · Neurotrophin · Recombinant

Introduction

Brain-derived neurotrophic factor (BDNF) plays a direct role in the regulation of multiple processes involved in neuronal cell growth, differentiation, and survival (Binder and Scharfman 2004; Ibáñez and Simi 2012; Leibrock et al. 1989; Numakawa et al. 2010). BDNF is natively expressed as proBDNF, which readily dimerizes intracellularly (Heymach and Shooter 1995; Jungbluth et al. 1994) and matures upon proteolytic cleavage of the pro peptide (Lee et al. 2001; Lu 2003; Mowla et al. 2001). BDNF signaling is mediated by two receptors, tropomyosin-related kinase B (TrkB) and p75 (Nagahara and Tuszynski 2011; Numakawa et al. 2010; Soppet et al. 1991; Teng et al. 2005). The TrkB receptor recognizes the mature dimerized form of BDNF and enhances neuronal growth and survival (Lu et al. 2005a; Numakawa et al. 2010), while the p75 receptor recognizes proBDNF causing neuronal cell death (Ibáñez and Simi 2012; Lu et al. 2005a; Numakawa et al. 2010; Teng et al. 2005). Disruption in BDNF signaling has been implicated in a number of neurodegenerative diseases, including Alzheimer's, Huntington's, and Parkinson's (Lu et al. 2005a; Nagahara and Tuszynski 2011;

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Zuccato and Cattaneo 2009). BDNF, and other members of the neurotrophin family, are also necessary to direct neuronal regeneration post-injury (Lu et al. 2005b; Menei et al. 1998). For these reasons, studies of BDNF function are critical for understanding neuronal biology and for the development of new therapeutic strategies for neurodegenerative diseases (Nagahara and Tuszynski 2011) and spinal cord injuries (Lynam et al. 2015). However, studies of BDNF function are limited by the cost of the protein from commercial sources.

BDNF is a cystine-knot protein (Binder and Scharfman 2004), containing a series of non-sequential disulfide bonds that make it difficult to express properly folded protein that retains the ability to homodimerize (Numakawa et al. 2010). Attempts to express BDNF in a variety of hosts have been limited by aggregate formation and low bioactivity (*Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*) (Burns et al. 2014, 2016; Fukuzono et al. 1995; Hoshino et al. 2002; Park and Shimizu 1996; Takeshita et al. 1996) or low yields (Sf21, RK_{13}, CHO, and HEK cells) (Burton 1993; Knusel et al. 1991; Meyer et al. 1992; Meyer et al. 1994), motivating the need for a better host for expressing BDNF at useful quantities with reasonable labor and material costs.

Brevibacillus choshinensis, a gram-positive bacterium, was originally isolated from soil and found to secrete large quantities of protein with low extracellular protease activity (Takagi et al. 1989). Because *B. choshinensis* is gram-positive, secreted protein can be purified without concern for contaminating endotoxins (Ilk et al. 2011). *B. choshinensis* has been used to successfully express a number of mammalian proteins, including the growth factors VEGF and NGF, which are, like BDNF, cystine-knot proteins (Sun and Davies 1995). In this work, we have expressed mature BDNF using *B. choshinensis*, preliminarily investigating the effects of temperature, medium composition, and culture time on bioactive protein production. We have found that culture conditions greatly impacted the production and purification of bioactive BDNF. This study demonstrates that *B. choshinensis* is capable of producing useful quantities of bioactive BDNF in laboratory-scale cultures.

Methods

Plasmid construction and expression strain

BDNF cDNA was purchased from Bioclon (San Diego, CA) and subcloned with a C-terminal 6× His-tag into the pNCMO2 *B. choshinensis* expression vector (Clontech cat# HB112), placing BDNF expression under the P2 promoter and adding an N-terminal R2L6 secretion tag, by In-fusion cloning (Clontech cat# 638909) (details in Expanded Methods). *B. choshinensis* competent cells were purchased from Clontech (cat# HB116). The strain used in this study has been genetically modified to remove any remaining proteolytic genes and the genes responsible for sporulation (Hanagata and Nishijyo 2010). Transformation of verified plasmids was performed by the Tris-PEG method, per manufacturer's instructions (Clontech). Single colonies were obtained after transformation, and protein expression was verified by western blotting (details below).

B. choshinensis culture conditions

Protein was expressed in 50-mL shake flasks in either TM medium (1% glucose, 1% polypeptone, 0.5% meat extract, 0.2% yeast extract, 0.001% $FeS0_4*7H_2O$, 0.001% $MnSO_4*4H_2O$, 0.0001% $ZnSO_4*7H_2O$, and 50 μg/mL neomycin) or 2SY medium (2% glucose, 4% soytone, 0.5% yeast extract, 0.015% $CaCl_2*7H_2O$, and 50 μg/mL neomycin), per manufacturer's recommendations (Clontech). Cultures were inoculated with a single colony from transformed *B. choshinensis* and grown overnight at 30 °C and 200 rpm in TM medium. Fifty milliliters of cultures was inoculated to an OD_{600} of 0.01 and grown either at 25 or 30 °C and 200 rpm. When needed, cultures were diluted in PBS to ensure accuracy of the OD_{600} measurements. Samples were removed or cultures collected at specified time points ranging up to 96 h at 30 °C and 120 h at 25 °C.

Protein purification

Cells were separated from BDNF-containing supernatants by centrifugation at 8000*g* for 10 min. Supernatants were supplemented with 10× MOPS buffer (250 mM MOPS pH 7.4, 5 M NaCl, and 40 mM imidazole) to a 1× final concentration to control pH, ionic concentration, and non-specific binding to Ni^{2+} Sepharose 6 Fast Flow (referred to as Ni^{2+} sepharose; GE Healthcare cat# 17-5318-06). Supernatants were then centrifuged at 16,000*g* for 20 min at 4 °C to remove any remaining insoluble material. Thirty-five milliliters of the recovered supernatants was then incubated with 200 μL of Ni^{2+} sepharose for 1 h with end-over-end rotation and recovered by centrifugation at 1000*g* for 5 min. The Ni^{2+} sepharose beads were washed three times in 1× MOPS buffer and eluted three times in 1× MOPS buffer with 300 mM imidazole, using centrifugation at 1000*g* for 5 min to separate the Ni^{2+} sepharose from the wash or elution buffer. All three elutions were combined and filter sterilized with a 0.22-μm PVDF low-binding filter (Millipore cat# SLGV013SL).

SDS-PAGE analysis, western blotting, and BDNF quantification

Samples for SDS-PAGE and western blotting were denatured and reduced, unless otherwise specified, then resolved on a 4–

20% SDS-PAGE gel. Gels were stained using GelCode Blue (Thermo Fisher cat# 24590), according to manufacturer's instructions. Gel-separated proteins were transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad cat# 161-0112) at 90 V for 70 min. BDNF was detected with either an anti-BDNF(N20) (Santa Cruz cat# sc-546) or HRP-conjugated anti-6× His (Cell Signaling cat# 9991) antibody. A HRP-linked anti-Rabbit antibody (Cell Signaling cat# 7074S) was used as a secondary antibody for both BDNF antibodies. All western blots were resolved with SuperSignal West Femto Maximum Sensitivity Substrate (ThermoFisher cat# 34094). Detailed western blotting methods are included in the Expanded Methods. A BDNF standard was purified to homogeneity under denaturing and reducing conditions and quantified by Bradford assay in comparison to a BSA standard (Fig. S1). BDNF concentrations in samples were quantified relative to a set of dilutions for this BDNF standard. Due to the denaturing conditions required for purification, the BDNF standard retained no bioactivity and thus could only be used as a western blot standard.

Proliferation assay for BDNF bioactivity

BDNF bioactivity was assayed via cell proliferation in mouse fibroblast 3T3 cells overexpressing the TrkB receptor (3T3-TrkB; generously provided by Jeffrey Sakamoto) (McCarty and Feinstein 1998). For the bioactivity assay, black, 96-well, clear bottom plates (Costar cat# 3904) were coated with 50 μL of 50 μg/mL poly-L lysine (Sigma, cat# P9155) for 1 h, washed three times with water, air-dried, and then coated with 50 μL of 2.5 μg/mL fibronectin (Sigma cat# F1141) overnight. Plates were washed with DMEM (Life Tech cat# 11965092) before cell plating. Cells were seeded at 2000 cells/well in defined medium (3:1 DMEM 11965:Ham's F12 (Life Tech cat# 11965092 and 11765047, respectively), 15 mM HEPES, 4 μM $MgCl_2$, 3 mM L-histidine, 10 μM ethanolamine, 1× ITS+1 (Sigma cat# I2521), 2 μM hydrocortisone, and 150 μg/mL G418 (ThermoFisher cat# 10131035)) for 3 h. To normalize for cell number, 10 μL (1/10th well volume) of AlamarBlue (ThermoFisher cat# DAL1285) was also included in the medium. AlamarBlue fluorescence was measured on a Biotek H4 plate reader (ex 570 nM, em 590 nM, filter 9.0) after 3 h. Initial fluorescence readings were used to normalize for differences in cell plating. The medium was then changed to BDNF-containing defined medium. Dilutions of each protein preparation were tested starting with 1% *v/v* purified protein and serially diluted 1:3 to 4.6×10^{-4}% *v/v*. For consistency in the medium composition and to control for effects of the elution buffer, a 1% *v/v* composition of elution buffer was used in all tests, equivalent to the concentration of buffer in the most concentrated protein samples. Commercial BDNF served as a positive control for protein activity (Affymetrix cat# 14-8366-62). This protein did not possess a His-tag and therefore was not used as a standard in the western blotting studies described above. A mock protein preparation of *B. choshinensis* carrying the empty pNCMO2 vector under each culture condition was used as a negative control. The medium was changed every 24 h for 4 days. After 4 days, AlamarBlue fluorescence was measured again, as before, and fold-difference in proliferation was calculated after background subtraction using the following equation:

$$\text{Fold Difference} = \frac{F_{\text{Final,Sample}} \Big/ F_{\text{Initial,Sample}}}{F_{\text{Final,Control}} \Big/ F_{\text{Initial,Control}}}$$

where F is the relative fluorescence intensity.

Images of cell proliferation were obtained by plating 20,000 3T3-TrkB cells/well in a 12-well plate. Plate coating was performed as described above with volumes of poly-L lysine and fibronectin adjusted to 400 μL/well. The assay was performed as described but with the omission of AlamarBlue readings. Images were taken prior to each medium change.

Statistical analysis

Two-tailed *t* test and two-way ANOVA analyses were performed using Graphpad Prism 6. BDNF bioactivities were compared by equivalence test using 90% confidence intervals to compare prepared BDNF to commercial BDNF. These analyses were done using one-way ANOVA with a Dunnett post hoc test in Graphpad Prism 6.

Results

Secretion of soluble BDNF from *B. choshinensis*

His-tagged BDNF was constitutively expressed under the P2 promoter in *B. choshinensis* using the pNCMO2 vector with an N-terminal secretion tag. Two types of growth media are suggested for expression in *B. choshinensis*, TM and 2SY. We monitored *B. choshinensis* growth and BDNF expression in both medium types over 72 h at 30 °C and 96 h at 25 °C (Fig. 1). Supernatant and cellular fractions were separated by centrifugation and analyzed by western blotting (Fig. 1 b, d, f, h), confirming the expression of BDNF, the presence of a His-tag, and the presence of soluble BDNF in the culture supernatants.

Protein recovered in both the supernatant and cellular fractions appeared at the size of mature BDNF, indicating removal of the *B. choshinensis* secretion tag (with tag 17.7 kDa and without tag 14.5 kDa; Fig. S2). Accumulation of BDNF without the secretion tag in the cellular fraction indicates that

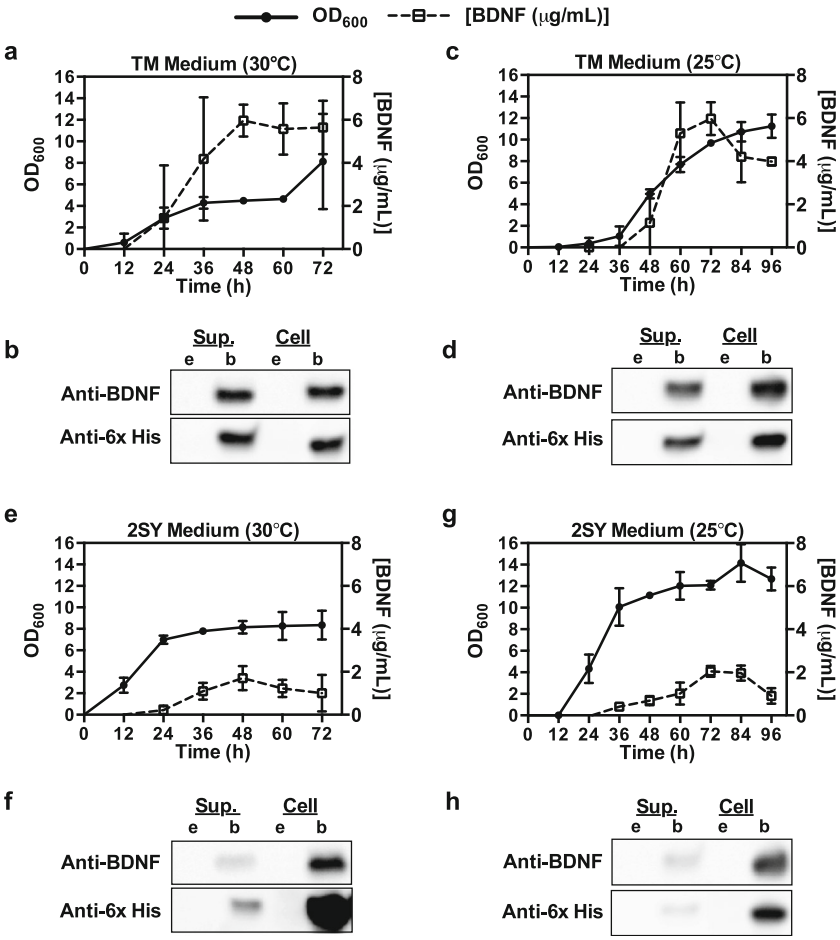

Fig. 1 *B. choshinensis* growth and BDNF expression. Growth of *B. choshinensis* and evaluation of BDNF secretion using two different medium types, TM (**a**, **c**) and 2SY (**e**, **g**), each at two different temperatures, 25 °C (**c**, **g**) and 30 °C (**a**, **e**). Growth was assayed using measurements of OD_{600} (*solid curves*). Protein expression was measured by western blotting (*dashed curves*). $N = 3$; *error bars* are ±1 SD. **b**, **d**, **f**, **h** Anti-BDNF (*top panel*) and anti-6× His (*bottom panel*) antibodies against samples from *B. choshinensis* cultures transformed with an empty vector (**e**) or the BDNF vector (**b**). Results are shown for the supernatant (*Sup.*) and cell pellet (*Cell*) fractions

BDNF is successfully secreted through the plasma membrane but then either remains associated with the cell wall or precipitates out of solution; in either case, it remains associated with the cellular fraction during centrifugation. The secretion tag (R2L6) is derived from the middle wall protein of *B. choshinensis* (Mizukami et al. 2010, 2015; Yamagata et al. 1987) and mediates secretion of as much as 0.8 g/L of recombinant, disulfide-bonded protein (Maehashi et al. 2010). The robustness of the secretion system further supports our conclusion that protein recovered in the cellular fraction has already passed the plasma membrane (i.e., minimal intracellular accumulation of BDNF with the secretion tag). That said, little BDNF could be recovered from the cellular fraction as soluble protein (Fig. S3, Cell–Sol.); as such, we did not further purify this protein or test its bioactivity.

Accounting for both the supernatant and cellular fractions, the total quantity of BDNF expression at 48 h (30 °C) or 72 h (25 °C) was similar for both medium types. However, medium type did change the distribution of BDNF between the supernatant and cell fractions, with a greater fraction of BDNF remaining in the supernatant in the TM cultures relative to the 2SY cultures (TM 45 ± 4% vs 2SY 13 ± 2%; $p < 0.05$, two-tailed t test; $N = 2$; Fig. 1). Medium type also affected the phase of growth in which the largest fraction of BDNF accumulated in the medium, with BDNF expression occurring largely prior to stationary phase in TM medium and during stationary phase in 2SY medium (Fig. 1). *B. choshinensis* growth in 2SY medium also led to a greater stationary phase OD_{600} at each temperature. These differences in cell growth and expression indicate lower per cell expression of BDNF in 2SY cultures.

Purification of BDNF by Ni^{2+} IMAC for bioactivity testing

Cultures were grown in triplicate, and BDNF was purified from culture supernatants by immobilized metal chromatography (IMAC) using Ni^{2+} sepharose. With this one-step protocol, we were able to purify sufficient quantities to test BDNF bioactivity, but the low stringency method resulted in variable protein recovery (Fig. 2a, Table S1) and purity (Fig. S4). Properly folded BDNF forms a cystine-knot, containing three disulfide bonds, leaving no free cysteines. Disulfide bond-mediated aggregates were observed in the culture supernatant (Fig. S5) and purified protein (Fig. S6). Bands at higher molecular weights suggest that contaminating proteins also co-purify with BDNF, perhaps mediated by non-specific

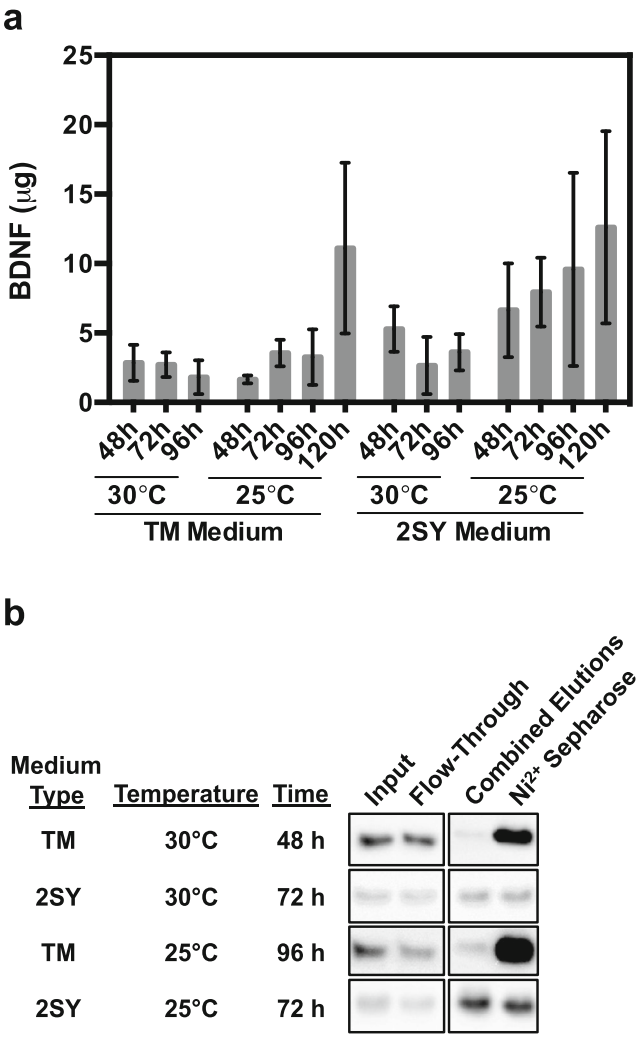

Fig. 2 Purification of BDNF. **a** Quantity of BDNF recovered by IMAC purification determined by western blotting with a standard curve. $N = 3$; *error bars* represent ±1 SD. **b** Representative western blots of samples taken during the purification process including input (35 mL), flow-through (35 mL), combined elutions (600 μL), and Ni^{2+} sepharose suspended in elution buffer (600 μL); volumes are the total volumes at each step in the process. 6.67 μL was loaded per lane. The Ni^{2+} sepharose suspended in elution buffer was analyzed to detect BDNF that remained associated with the Ni^{2+} sepharose after elution. Samples for each culture condition (*each row*) were run on a single western. Images show the purification fractions for samples from the earliest time at which maximal protein bioactivity was measured for the given condition (see Fig. 3)

interactions with BDNF aggregates. Higher fractional yields were obtained for lower initial protein loadings, indicating saturation of the Ni^{2+} sepharose; however, the maximum quantity of BDNF recovered (18 μg) was well below the theoretical binding capacity of the Ni^{2+} sepharose (8 mg). Still, some BDNF did not bind to the Ni^{2+} sepharose (Fig. 2b, Flow-Through). This suggests that protein is expressed without an accessible His-tag, perhaps due to aggregate formation. Additionally, some BDNF bound to the Ni^{2+} sepharose but did not elute with imidazole (Fig. 2b, Ni^{2+} Sepharose). This indicates fouling of the Ni^{2+} sepharose by protein precipitation (GE Healthcare 2016).

Recombinant BDNF is bioactive

BDNF bioactivity was measured via a proliferation assay, using mouse NIH3T3 fibroblasts engineered to respond to BDNF through overexpression of TrkB (McCarty and Feinstein 1998). Activation of TrkB signaling with BDNF causes a mitogenic response in the 3T3-TrkB cells (McCarty and Feinstein 1998). In the absence of serum and BDNF, 3T3-TrkB cells do not proliferate but remain generally healthy and metabolically active (Figs. 3a and S7, (−) Control). Cell proliferation in the presence or absence of BDNF in serum-free medium was monitored over the course of 4 days with the medium changed daily. After 4 days, AlamarBlue was used to assess cell number, and proliferation was determined by the increase in cell number of BDNF-treated wells relative to control wells (Fig. 3b). Because BDNF samples were not dialyzed after elution from the Ni^{2+} sepharose, the imidazole concentration present at 1% *v/v* of purified BDNF (3 mM) was made up in cultures exposed to diluted BDNF samples. A single EC_{50} curve was fit to bioactivity data of BDNF protein prepared from three replicate *B. choshinensis* cultures for each culture condition and time (Fig. S8).

Changes in bioactivity with culture time were investigated for each medium type, culturing for a minimum of 48 h and up to 120 h at 25 °C or 96 h at 30 °C. We did not test bioactivities for protein purified from cultures earlier than 48 h because these were the earliest cultures for which BDNF levels were maximal under some conditions (Fig. 1). We took samples every 24 h until at least three time-points were taken after BDNF levels had reached a maximum, resulting in the longer sampling period for cultures at 25 °C relative to 30 °C. BDNF bioactivity was compared to a bioactive, commercial BDNF (Fig. 3b). Only protein obtained from cultures grown in 2SY medium had comparable bioactivity to the commercial protein (Fig. 3b), indicating that, at least when using our current purification protocol, cultures grown in TM medium produced a lower fraction of bioactive BDNF in the supernatants. Furthermore, in 2SY medium, we observed that EC_{50} values improved with culture time (Fig. 3b). This suggests that, as in TM medium, not all of the protein produced in 2SY medium is bioactive and that the fraction of bioactive protein present in the supernatant increases with time. We confirmed that bioactivity was due specifically to protein produced from the BDNF-containing vector, as shown by our empty vector controls (Fig. 3a, c). Decreasing 3T3 cell numbers with time in the empty-vector controls reflect some eventual cytotoxicity due to the absence of serum and BDNF.

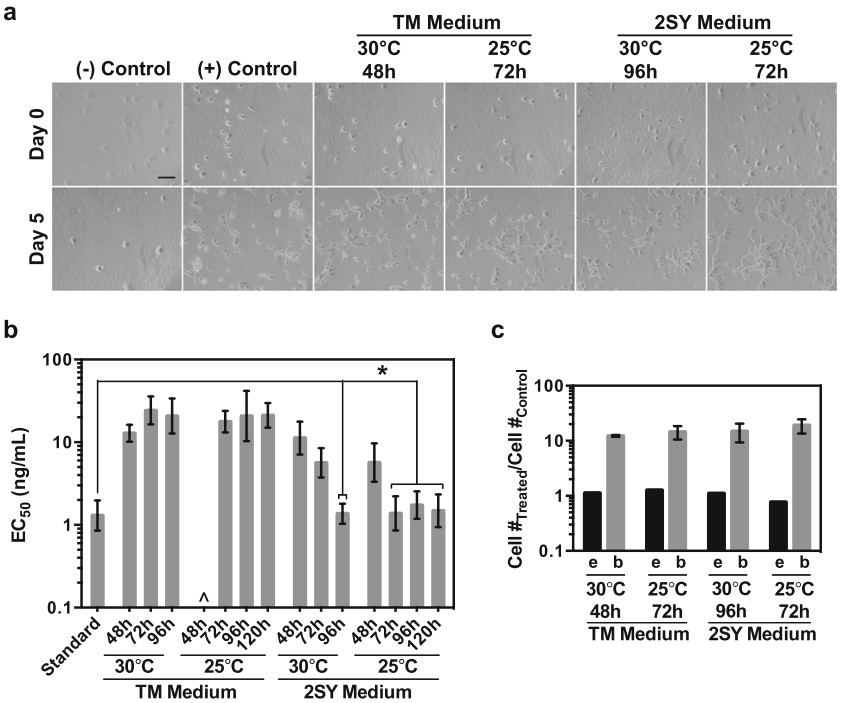

Fig. 3 Bioactivity of BDNF. BDNF specific activity was measured by proliferation assay. **a** Purified protein samples were added at 1% *v/v* to serum-free defined medium. Recombinant BDNF at 100 ng/mL and wells without BDNF were used as (+) and (−) controls, respectively. Shown are representative images from days 1 to 5 of the same location within each well. Images are shown for cells exposed to protein purified from the earliest *B. choshinensis* cultures at which maximal protein bioactivity was measured for the given medium and temperature conditions. *Scale bar* represents 100 μm. Additional images are included in Fig. S7. Details on image capture are included in Expanded Methods. **b** EC_{50} values measured for BDNF prepared from three replicate *B. choshinensis* cultures for each expression condition and fit with a single dose-response curve (Fig. S8); (^) EC_{50} was not reached at the highest concentration tested (1% *v/v*); *error bars* indicate ±1 SD of the dose-response curve fit; (*) bioactivity is comparable to commercially available BDNF by equivalence test using 90% confidence intervals (Fig. S9). **c** Comparison of bioactivity of protein purified from cultures transfected with an empty vector (e) or the BDNF vector (b)

Discussion

In this work, we demonstrated that BDNF purified from *B. choshinensis* cultures was biologically active. In testing a variety of culture conditions and times, we identified conditions that provided sufficient yields for subsequent bioactivity studies. Use of *B. choshinensis* as the host mitigated the principal difficulty in achieving expression of bioactive BDNF, its cystine-knot structure, which can lead to intermolecular disulfide linkages, incorrect intramolecular disulfide linkages, and aggregate formation (Binder and Scharfman 2004; Burns et al. 2014; Meyer et al. 1994; Shimizu et al. 1996). Nonetheless, despite our yields of bioactive protein, considerable amounts of BDNF produced still formed disulfide-bonded aggregates (Fig. S5). To improve BDNF yields, additional optimization of expression conditions should focus on reducing the formation of aggregates, either during the expression process or after accumulation in the culture supernatant, which will likely have the concomitant benefits of improving recovery of BDNF and reducing the concentration of contaminants that co-purify with BDNF.

Aggregates were observed in all conditions; the quantity of monomeric BDNF in the supernatant was greater in TM cultures. However, in our experiments, we were unable to obtain a sample of BDNF from TM cultures with comparable activity to the commercial standard. Our purification by Ni^{2+} sepharose selectively enriched monomeric BDNF (Fig. S6); nonetheless, the purified protein was not all bioactive BDNF, since the protein recovered from TM cultures had lower specific bioactivity than protein recovered from 2SY cultures at equivalent culture times and temperatures (Fig. 3b). These results suggest that the fraction of properly folded BDNF varies between TM and 2SY cultures. It may still be possible to recover a more active fraction of BDNF from TM cultures, but, given the degree to which aggregation may have precluded purification of bioactive BDNF in TM samples, our current results suggest 2SY medium is a better choice for future optimization.

It may be possible to remove aggregates by leveraging the stability of BDNF at pH values as low as 4 (Tanaka and Kumano 2000); at low pH, aggregates should precipitate and can then be removed by centrifugation, similar to what

has been done with EGF purification (Miyauchi et al. 1999). Monomeric BDNF could then be purified using a variety of other techniques, including IMAC, as we performed here, or by cationic exchange, anionic exchange, or reversed-phase chromatographic methods, as have been studied elsewhere (Burton 1993; Jungbluth et al. 1994; Rosenfeld and Benedek 1993).

Reducing the culture temperature from 30 to 25 °C resulted in a greater fraction of biologically active BDNF at an earlier culture time in 2SY medium (72 vs 96 h, Fig. 3b). In other expression systems (i.e., *E. coli*), reductions in culture temperature are known to improve the expression of recombinant proteins, by reducing the rate of translation (Vasina and Baneyx 1997) and the propensity for protein aggregation (Baldwin 1986; Schellman 1997). In 2SY medium, the reduction in culture temperature also increased the stationary phase OD_{600} and decreased the rate of BDNF accumulation in the culture supernatant, indicating a marked reduction in the per cell expression of BDNF. While not explored here, the lower rate of BDNF expression (by reduction in temperature) does correspond with the ability to produce biologically active BDNF. This could be further supported through experiments using a lower strength promoter for BDNF expression (Onishi et al. 2013). Reduction to 20 °C resulted in a significantly longer lag phase, especially in TM medium, and was not explored further due to practical culture time limitations (Fig. S10). However, altering the culture conditions (i.e., beginning with a more concentrated inoculum) to reduce culture duration would allow investigation of expression at temperatures below 25 °C and could be beneficial in the production and recovery of biologically active BDNF.

For our best conditions (2SY medium at 25 °C for 72 h), we were able to purify 264 ± 82 μg/(L of culture) of biologically active BDNF. We consider these our best conditions as they resulted in (i) BDNF with the highest bioactivity, (ii) the highest yield of BDNF, and (iii) the minimum culture time. The quantity of recoverable BDNF did not significantly increase at times >72 h in 2SY medium at 25 °C, but we did observe an increase in the variability of purified BDNF with time, with several cultures producing >500 μg/L of biologically active BDNF. The yield of BDNF is greater than or of the same magnitude as other expression systems (Fukuzono et al. 1995; Knusel et al. 1991; Meyer et al. 1994; Rosenthal et al. 1991). In addition, the use of a microbial system offers both cost (<$10/L medium) and technical advantages (standard microbial culturing techniques) over higher complexity expression systems (Knusel et al. 1991; Meyer et al. 1994; Rosenthal et al. 1991) or methods that require refolding BDNF (Collins et al. 1993). Recently, ~1 mg/L expression of BDNF was demonstrated in yeast after the engineering of BDNF to facilitate protein folding (Burns et al. 2014, 2016); it is conceivable that using a modified BDNF sequence for expression in *B. choshinensis* could also significantly increase expression of biologically active BDNF in this host.

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Compliance with ethical standards This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest.

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