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Modulation of neuronal differentiation by CD40 isoforms

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Abstract

Neuron differentiation is a complex process involving various cell-cell interactions, and multiple signaling pathways. We showed previously that CD40 is expressed and functional on mouse and human neurons. In neurons, ligation of CD40 protects against serum withdrawal-induced injury and plays a role in survival and differentiation. CD40 deficient mice display neuron dysfunction, aberrant neuron morphologic changes, and associated gross brain abnormalities. Previous studies by Tone and colleagues suggested that 5 isoforms of CD40 exist with 2 predominant isoforms expressed in humans: signal-transducible CD40 type I and a C-terminal truncated, non-signal-transducible CD40 type II. We hypothesized that differential expression of CD40 isoform type I and type II in neurons may modulate neuron differentiation. Results show that adult wild-type, and CD40^{-/-} deficient mice predominantly express CD40 type I and II isoforms. Whereas adult wild-type mice express mostly CD40 type I in cerebral tissues at relatively high levels, in age and gender-matched CD40^{-/-} mice CD40 type I expression was almost completely absent; suggesting a predominance of the non-signal-transducible CD40 type II isoform. Younger, 1 day old wild-type mice displayed less CD40 type I, and more CD40 type II, as well as, greater expression of soluble CD40 (CD40L/CD40 signal inhibitor), compared with 1 month old mice. Neuron-like N2a cells express CD40 type I and type II isoforms while in an undifferentiated state, however once induced to differentiate, CD40 type I predominates. Further, differentiated N2a cells treated with CD40 ligand express high levels of neuron specific nuclear protein (NeuN); an effect reduced by anti-CD40 type I siRNA, but not by control (non-targeting) siRNA. Altogether these data suggest that CD40 isoforms may act in a temporal fashion to modulate neuron differentiation during brain development. Thus, modulation of neuronal CD40 isoforms and CD40 signaling may represent important therapeutic modalities for neurodegenerative and neurodevelopmental disorders, as well as, for enhancement of neurogenesis.

Keywords

CD40; CD40 isoform; CD154; CD40L; CD40 ligand; neuronal differentiation; neurodevelopment; neuronal cells

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INTRODUCTION

Neuronal differentiation during development or in adult stem cell populations is a complex and tightly regulated process known to involve myriad soluble factors, cell-cell, and cell-extracellular matrix interactions¹. CD40 receptor (CD40) is a 45 – 50 kDa receptor that is a member of the tumor necrosis factor receptor (TNFR) superfamily^{2,3}. CD40 expression and function has been described on a variety of cell types^{4–7}, including neurons⁸. Activation of the CD40 pathway is well-known to promote proliferation, survival, differentiation and maturation in a variety of immune and other cell types^{9–20}.

We previously showed that ligation of CD40 by CD40 ligand (CD40L) protected neuronal cells from NGF- β or serum withdrawal-induced injury and promoted differentiation⁸. In addition, we previously found that adult CD40 deficient mice demonstrated abnormal neuronal dysfunction, morphology and gross brain abnormality. Seminal investigations by Tone and colleagues suggested that 5 distinct isoforms of CD40 are expressed with 2 isoforms predominating in humans and mice: signal-transducible CD40 type I and a C-terminal truncated isoform, non-signal-transducible, CD40 type II²¹. Our studies presented here investigate whether differential expression of CD40 type I and type II isoforms by neurons may modulate neuron differentiation. Differential expression of cerebral CD40 isoforms was observed and occurred in a temporal manner during postnatal murine brain development. In addition, CD40L signaling, mediated by CD40 type I, may enhance neuron differentiation; a process potentially downregulated by both CD40 type II²¹ and soluble CD40 receptor (sCD40) proteins^{22–24}.

MATERIALS AND METHODS

Animals

CD40 disrupted mice were purchased from the Jackson Laboratory (Bar Harbor, ME). These mice and background control mice were then killed at 1 day, 1 month or 14 months of age for pathologic analysis. Animals were given food and water ad libitum. They were housed and maintained in the University of South Florida (USF), College of Medicine Animal Facility, and all experiments were in compliance with protocols approved by the USF Institutional Animal Care and Use Committee.

Cell culture

N2a (neuroblastoma) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). N2a cells were grown in complete Eagle's minimum essential medium (EMEM) and were induced to differentiate by reducing fetal bovine serum from 10% to 1% and the addition of 3 mM dibutyryl cAMP for 48 – 72 h.

Immunohistochemistry staining analysis

Mice were anesthetized with isoflurane and transcardially perfused with ice-cold PBS, the brains were rapidly isolated and quartered using a mouse brain slicer (Muromachi Kikai, Tokyo, Japan). The first and second anterior quarters were homogenized for Western blot analysis, and the third and fourth posterior quarters were used for microtome or cryostat sectioning. The posterior quarters of the brains for frozen sectioning were fixed in 4% paraformaldehyde in PBS at 4°C for 24 h, and then change to 10%, 20% and 30% sucrose in PBS every 24 h. Five coronal sections from each brain (25 μ m thicknesses) were cut with a 150 μ m interval, and did immunofluorescent staining. Briefly, the sections were blocked in 10% horse serum (ATCC) for 1 h, and incubated in Fluorescein Isothiocyanate (FITC)-conjugated hamster anti-mouse CD40 (N-CD40) (1:100; Pharmingen, San Jose, CA), rabbit anti-CD40 (C-CD40) (1:200; Stressgen, San Diego California) antibodies overnight, wash

with PBS 3 times, 10 minutes each time. For C-CD40, then incubated in Alexa Fluor® 555 donkey anti-rabbit IgG (1:500; Invitrogen, Carlsbad, CA) for 45 minutes, wash again, and mounted with VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA).

Western analysis

For detection of CD40 isoforms *in vitro*, N2a cells were plated in six-well tissue culture plates at 5×10^5 cells/well and differentiated as described above. After treatment, cell lysates were prepared, and Western blotting was performed as described previously²⁵. Briefly, membranes were hybridized with either anti-N-terminal CD40, anti-C-terminal CD40, or anti-actin (1:4000, Sigma) antibodies followed by incubation with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. The protein bands were detected with a Super Signal west Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) and BIOMAX-MR film (Eastman Kodak Co., Providence, RI). For detection of CD40 isoforms *in vivo*, brain homogenates were prepared from the frontal quarters of the brains, and Western blotting was performed as described above.

Small interfering RNA mediated gene silencing

N2a cells were transfected with siRNA pre-designed to knock-down murine CD40 type I mRNA (Dharmacon Inc. Lafayette, Colorado). N2a cells were seeded in 24-well plates and cultured until they reached 70% confluence. The cells were then transfected with 50–200 nM CD40 type I siRNA or anti-green fluorescent protein (GFP; non-targeting control; Dharmacon) using Code-Breaker transfection reagent (Promega, Madison, Wisconsin) and cultured for an additional 18 h in serum-free MEM. Transfection efficiency was determined to be greater than 80% (data not shown) using no-RISC siGLOW (fluorescently labeled non-functional siRNA; Dharmacon). The cells were allowed to recover for 24 h in complete medium (MEM 10% FBS) before treatments. The cells were evaluated by immunocytochemistry staining and Western blot analyses for expression of CD40.

Statistical analysis

All quantitative data were analyzed by analysis of variance (ANOVA) followed by post-hoc comparisons of means using Bonferroni's or Dunnett's T3 methods where appropriate, and t-test for independent samples was used to assess significance for single mean comparisons. Alpha levels were set at 0.05 for all analyses, which were performed using SPSS for Windows release 10.0.5.

RESULTS

CD40^{-/-} deficient mice display reduced CD40 type I isoform

We previously reported that the CD40-CD40L pathway may play a role in differentiation and survival of neurons. In addition, Tone and colleagues reported that in murine macrophages and dendritic cells, down-regulation of CD40 signaling can occur by expression of non-signal transducible type II CD40 isoform²¹. To determine whether differential expression of CD40 isoforms plays a role in neuron differentiation, we first monitored the effect of disruption of CD40 type I signaling *in vivo* by analysis of the expression of CD40 isoforms in cortex and CA1 regions of gender-matched wild-type and CD40^{-/-} mice at 14 month of age. As N-terminal CD40 antibodies are expected to bind both type I and type II isoforms of CD40, and C-terminal CD40 antibodies bind only longer signal-transducible CD40 isoforms, predominately type I in our models, the differential expression of CD40 isoforms type I and II can be analyzed. Results show a dramatic reduction in expression of the signal-transducible CD40 type I in the cortex and CA1 brain

regions of CD40^{-/-} mice when compared to age, gender, and strain-matched controls (wild-type mice; Figure 1A and B). Similar results are obtained by Western blotting analysis (Figure 1C).

Type I CD40 isoform expression associated with neuronal maturation in mice

To further determine the role of differential expression of CD40 isoforms in neuron differentiation we examined differential isoform expression during neurodevelopment. Murine neurodevelopment continues through birth but is considered definitely complete at 12 months of age, however considerable changes in neurodevelopment also occur in the postnatal period between birth and 1 month²⁶, thus we examined differential expression of CD40 isoforms in wild-type mice at these timepoints. Mice at 1 month of age displayed increased CD40 type I isoform in cortex and CA1 regions by Immunohistochemistry staining as compared with younger wild-type mice (Figure 2A and B). As shown in Figure 2C and D (and supplementary Figure 1A and B), by Western blotting analysis of brain homogenates, CD40 type I isoform was increased and CD40 type II was decreased in 1 month old wild-type mice compared to 1 day old wild-type mice. The CD40 type I to type II ratio of protein expression in brain homogenates is increased at the 1 month timepoint relative to 1 day old mice. Moreover, consistently, a CD40 pathway antagonist molecule, soluble CD40 (sCD40)²², was also decreased in brain homogenates (supplementary Figure 2C).

Type I CD40 isoform associated with differentiation in neuron-like N2a cells

Our *in vivo* findings lead us to next monitor differential expression of CD40 isoform type I and II in undifferentiated and differentiated neuron-like N2a cells. Differentiated N2a cells, by immunohistochemistry staining (Figure 3A) and Western blot analysis (Figure 3B), display increased CD40 type I expression and decreased CD40 type II when compared with undifferentiated N2a cells. Further the ratio of CD40 type I to type II is increased in differentiated N2a cells (Figure 3C and D; supplementary Figure 2A and B). As expected from our *in vivo* data, soluble CD40 was decreased in differentiated N2a cell media (supplementary Figure 2C). Differentiation of N2a cells was confirmed by expression of NeuN monitored by immunohistochemistry staining and morphology analyses (data not shown).

siRNA knockdown of CD40 type I reduces differentiation of neuron-like N2a cells

Finally, to determine the functional effects of CD40 signal regulation by differential isoform expression on neuron-like N2a cells, anti-CD40 type I siRNA was used to pre-treat N2a cells before application of differentiation media. As shown in Figure 4A and B, expression of CD40 type I and type II was monitored by immunohistochemistry staining and Western blotting analyses to confirm specific knockdown of CD40 type I isoform. Furthermore, anti-CD40 type I siRNA-treated N2a cells displayed similar expression of neuron specific NeuN expression by immunohistochemistry staining, compared with control, non-targeting siRNA-treated N2a cells (Figure 4C, left panels). However, after ligation of CD40 with CD40L, anti-CD40 type I siRNA-treated N2a cells displayed dramatically decreased neuron-like morphology, and NeuN expression (Figure 4C, right panels).

DISCUSSION

We found that in adult wild-type mice at 14 months of age the predominate isoform of CD40 expressed is signal transducible CD40 type I, whereas CD40^{-/-} mice appear to express only a mutated non-signal transducible CD40 isoform type II; lacking the C-terminal antibody binding epitope (Figure 1). In CD40^{-/-} mice, the dramatic reductions in expression of CD40 type I, as well as, enhanced expression of essentially a null receptor for

CD40L (CD40 type II) occurs vis-à-vis the targeted neomycin cassette interruption of exon 3 of CD40; comprising a mutation of the ligand binding portion of the extracellular domain²⁶. The insertion of this neomycin cassette is likely responsible for the separation of CD40 type II at a higher molecular weight range, relative to wild-type CD40 type II, in CD40^{-/-} brain homogenates by Western analysis (Figure 1). Thus, these data suggest an extreme compromise of both external and internal signal-transducing functions of CD40 in these mice. Moreover there is likely disruption in membrane bound CD40L signaling due to lack of proper CD40L-CD40 binding. These data are consistent with our prior observations demonstrating reduced CD40 signal transduction and co-occurring neuron loss, aberrant morphology, and gross brain abnormalities in the cortices and olfactory bulbs of CD40^{-/-} mice [8].

Interestingly, where 14 month old wild-type mice expressed predominantly CD40 type I, younger 1 day and 1 month old wild-type mice expressed multiple isoforms, predominantly type I and II (Figure 2). The temporal pattern of CD40 isoform expression suggested that CD40 type I may be associated with more mature stages of neurodevelopment whereas high levels of CD40 type II may exist in the early postnatal period (Figure 2). These data point towards a facilitation of CD40L/CD40 signaling occurring with progressing neurodevelopment. One possible explanation for the differential expression of CD40 isoforms, analogous to its role in peripheral immune system, could lie with the ability of CD40 to promote and maintain neuronal differentiation during neurodevelopment. Clearly CD40^{-/-} mice, lacking functional CD40 display developmental abnormalities [8]. Further, during early neurodevelopment, the process of neurogenesis may require tight regulation of CD40 signaling, negative regulation of which may occur *via* expression of CD40 isoform type II. Accordingly we next investigated differential expression of CD40 isoforms in neuron-like N2a cells and found an expression pattern dependent on state of differentiation (Figure 3). However, to determine whether CD40 signaling and isoform modulation could functionally affect differentiation of neuron-like N2a cells we specifically silenced CD40 type I using anti-CD40 type I siRNA (Figure 4A and B). Interestingly, N2a cells in differentiation media, expressed low levels of neuron specific marker NeuN after 48 hours in culture, however co-treatment with CD40L lead to marked expression of NeuN; an effect blocked by pre-treatment with anti-CD40 type I siRNA; but not by non-targeting control siRNA (Figure 4C). Again these data support our hypotheses that the CD40 pathway can modulate neuron differentiation, and that differential expression of CD40 isoforms, in combination with other factors such as soluble CD40, may act to influence neuronal differentiation during neurodevelopment. Furthermore, these data provide therapeutic targets for the potential modulation of neurogenesis and comprise important implications for future development of treatment and screening modalities for both neurodevelopmental, as well as, neurodegenerative disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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REFERENCES

1. Mehler MF. Mechanisms regulating lineage diversity during mammalian cerebral cortical neurogenesis and gliogenesis. *Results Probl. Cell Differ.* 2002; 39:27–52. [PubMed: 12357985]
2. Grimaldi JC, Torres R, Kozak CA, Chang R, Clark EA, Howard M, Cockayne DA. Genomic structure and chromosomal mapping of the murine CD40 gene. *J Immunol.* 1992; 149:3921–3926. [PubMed: 1281194]
3. Torres RM, Clark EA. Differential increase of an alternatively polyadenylated mRNA species of murine CD40 upon B lymphocyte activation. *J Immunol.* 1992; 2:620–626. [PubMed: 1370315]
4. van Kooten C, Gaillard C, Galizzi JP, Hermann P, Fossiez F, Banchereau J, Blanchard D. B cells regulate expression of CD40 ligand on activated T cells by lowering the mRNA level and through the release of soluble CD40. *Eur. J. Immunol.* 1994; 24:787–792. [PubMed: 7512028]
5. Mach F, Schönbeck U, Sukhova GK, Bourcier T, Bonnefoy JY, Pober JS, Libby P. Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A.* 1997; 5:1931–1936. [PubMed: 9050882]
6. Brouty-Boyé D, Pottin-Clémenceau C, Doucet C, Jasmin C, Azzarone B. Chemokines and CD40 expression in human fibroblasts. *Eur. J. Immunol.* 2000; 3:914–919.
7. Sugiura T, Kawaguchi Y, Harigai M, Takagi K, Ohta S, Fukasawa C, Hara M, Kamatani N. Increased CD40 expression on muscle cells of polymyositis and dermatomyositis: role of CD40-CD40 ligand interaction in IL-6, IL-8, IL-15, and monocyte chemoattractant protein-1 production. *J. Immunol.* 2000; 12:6593–6600. [PubMed: 10843719]
8. Tan J, Town T, Mori T, Obregon D, Wu Y, DelleDonne A, Rojiani A, Crawford F, Flavell RA, Mullan M. CD40 is expressed and functional on neuronal cells. *EMBO J.* 2002; 4:643–652. [PubMed: 11847112]
9. Zhou M, Gu L, Holden J, Yeager AM, Findley HW. CD40 ligand upregulates expression of the IL-3 receptor and stimulates proliferation of B-lineage acute lymphoblastic leukemia cells in the presence of IL-3. *Leukemia.* 2000; 3:403–411. [PubMed: 10720134]
10. Kilger E, Kieser A, Baumann M, Hammerschmidt W. Epstein-Barr virus-mediated B-cell proliferation is dependent upon latent membrane protein 1, which simulates an activated CD40 receptor. *EMBO J.* 1998; 6:1700–1709. [PubMed: 9501091]
11. Hasbold J, Hong JS, Kehry MR, Hodgkin PD. Integrating signals from IFN-gamma and IL-4 by B cells: positive and negative effects on CD40 ligand-induced proliferation, survival, and division-linked isotype switching to IgG1, IgE, IgG2a. *J. Immunol.* 1999; 8:4175–4181. [PubMed: 10510353]
12. Péguet-Navarro J, Dalbiez-Gauthier C, Moulon C, Berthier O, Réano A, Gaucherand M, Banchereau J, Rousset F, Schmitt D. CD40 ligation of human keratinocytes inhibits their proliferation and induces their differentiation. *J. Immunol.* 1997; 1:144–152.
13. Fischer HG, Reichmann G. Brain dendritic cells and macrophages/microglia in central nervous system inflammation. *J Immunol.* 2001; 4:2717–2126. [PubMed: 11160337]
14. Flores-Romo L, Björck P, Duvert V, van Kooten C, Saeland S, Banchereau J. CD40 ligation on human cord blood CD34+ hematopoietic progenitors induces their proliferation and differentiation into functional dendritic cells. *J. Exp. Med.* 1997; 2:341–349. [PubMed: 9016882]
15. Markowicz S, Engleman EG. Granulocyte-macrophage colony-stimulating factor promotes differentiation and survival of human peripheral blood dendritic cells in vitro. *J. Clin. Invest.* 1990; 3:955–961. [PubMed: 2179270]
16. Hata Y, Uchino J, Sasaki F, Une Y, Naito H, Sato K, Kukita K, Sano H, Kasai Y, Tsukada Y, et al. Effect of anti-alpha-fetoprotein serum on human hepatoblastoma. *J. Pediatr. Surg.* 1984; 5:573–576. [PubMed: 6209380]
17. Bachmann MF, Schwarz K, Wolint P, Meijerink E, Martin S, Manolova V, Oxenius A. Cutting edge: distinct roles for T help and CD40/CD40 ligand in regulating differentiation of proliferation-competent memory CD8+ T cells. *J. Immunol.* 2004; 4:2217–2221. [PubMed: 15294930]

18. Concha M, Vidal MA, Moreno I, Salem C, Figueroa CD, Schmitt D, Péguet-Navarro J. Evidence for modulation of human epidermal differentiation and remodelling by CD40. *Br. J. Dermatol.* 2003; 6:1105–1114. [PubMed: 12828736]
19. Townsend KP, Town T, Mori T, Lue LF, Shytte D, Sanberg PR, Morgan D, Fernandez F, Flavell RA, Tan J. CD40 signaling regulates innate and adaptive activation of microglia in response to amyloid beta-peptide. *Eur. J. Immunol.* 2005; 3:901–910. [PubMed: 15688347]
20. Rosenzweig M, Jourquin F, Tailleux L, Gluckman JC. CD40 ligation and phagocytosis differently affect the differentiation of monocytes into dendritic cells. *J. Leukoc. Biol.* 2002; 6:1180–1189. [PubMed: 12488500]
21. Tone M, Tone Y, Fairchild PJ, Wykes M, Waldmann H. Regulation of CD40 function by its isoforms generated through alternative splicing. *Proc. Natl. Acad. Sci. U S A.* 2001; 4:1751–1756. [PubMed: 11172023]
22. Nemoto E, Tada H, Shimauchi H. Disruption of CD40/CD40 ligand interaction with cleavage of CD40 on human gingival fibroblasts by human leukocyte elastase resulting in down-regulation of chemokine production. *J. Leukoc. Biol.* 2002; 72:538–545. [PubMed: 12223522]
23. van Kooten C. Blockade of costimulatory pathways of T-cell activation: the solution to acute and chronic rejection? *Curr. Opin. Nephrol. Hypertens.* 1999; 6:675–680. [PubMed: 10630812]
24. Fanslow WC, Anderson DM, Grabstein KH, Clark EA, Cosman D, Armitage RJ. Soluble forms of CD40 inhibit biologic responses of human B cells. *J. Immunol.* 1992; 149:655–660. [PubMed: 1378075]
25. Tan J, Town T, Paris D, Mori T, Suo Z, Crawford F, Mattson MP, Flavell RA, Mullan M. Microglial activation resulting from CD40-CD40L interaction after beta-amyloid stimulation. *Science.* 1999; 5448:2352–2355. [PubMed: 10600748]
26. Clinton M, Manson J, McBride D, Miele G. Gene expression changes during murine postnatal brain development. *Genome Biol. RESEARCH0005.* 2000; 3 Epub.
27. Kawabe T, Naka T, Yoshida K, Tanaka T, Fujiwara H, Suematsu S, Yoshida N, Kishimoto T, Kikutani H. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity.* 1994; 3:167–178. [PubMed: 7534202]

Figure 1.

Characterization of anti-N-terminal CD40 antibody (N-CD40 Ab) and anti-C-terminal CD40 antibody (C-CD40 Ab) in CD40 deficient (CD40^{-/-}) mice. (A, B) Immunofluorescent staining analysis was performed on vibratome sections of CD40^{-/-} mouse (left) or wild-type mouse (CD40^{+/+}, right) brains by C-CD40 Ab or N-CD40 Ab as indicated. N-CD40 Ab positive cells (green) are neurons for both CD40^{-/-} and CD40^{+/+} mice. However, C-CD40 Ab positive cells (red) are neurons for CD40^{+/+} mouse, but not for CD40^{-/-} mouse. (C) CD40 isoform expression in brain homogenates from both CD40^{-/-} and CD40^{+/+} mice by Western blotting analysis as indicated.

Figure 2.

CD40 isoform expressions *in situ* on neurons. Immunofluorescent staining analysis was performed *in situ* on neurons from mice at 1 day old and 1 month of age by N-CD40 Ab (A) or C-CD40 Ab (B) as indicated. Western blotting analysis of CD40 isoform expression in brain homogenates from mice at 1 day old and 1 month of age by N-CD40 Ab (C) or C-CD40 Ab (D), with $n = 6$ for each group.

Figure 3.

CD40 isoforms are expressed by neuron-like N2a cells. (A) Immunofluorescent staining by N-CD40 Ab or C-CD40 Ab was performed on cultured N2a cells prior to and after differentiation as indicated. N-CD40 Ab positive cells (green) are neuron-like cells for both differentiated N2a cells (Diff.) and undifferentiated N2a cells (Undiff.). However, C-CD40 Ab positive cells (red) are neuron-like cells for Diff. N2a cells, but not for Undiff. N2a cells. Western blotting analysis of CD40 isoform expression in N2a cells prior to and after differentiation by N-CD40 Ab (B) or C-CD40 Ab (B), with $n = 4$ for each condition presented.

Figure 4.

Anti-CD40 type I siRNA inhibits CD40L-induced neuronal differentiation. (A) Expression of CD40 type I and inhibition by transfection anti-CD40 type I siRNA, but not non-targeting control anti-GFP siRNA (Control), were confirmed by immunochemistry staining analysis in differentiated (48 h) cultures of N2a cells. (B) The efficiency for siRNA knockdown of CD40 type I is confirmed by Western blot analysis. Densitometry analysis shows the band density ratio of CD40 to actin as indicated in the panel to the right. One-way ANOVA revealed significant differences between anti-CD40 type I siRNA-transfected cells and non-targeting siRNA control treated cultures on expression of CD40 type I to actin ($**P > 0.001$). Similar data were obtained in three independent experiments. (C) In addition, N2a cells maintained in differentiation media for 48 h displayed low levels of neuron specific NeuN (upper left panel), however upon pretreatment with CD40L expression of NeuN increases dramatically (upper right panel). Expression of NeuN was decreased by transfection with anti-CD40 type I siRNA in N2a cells (lower left panel), not non-targeting control siRNA (lower right panel).