NeuroReport 6, 1326–1330 (1995)

Glutamate regulation of GDNF gene expression in the striatum and primary striatal astrocytes

Angela Ho, Andrea C. Gore, Cynthia Shannon Weickert and Mariann Blum[CA]

Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, One Gustave Levy Place, New York, NY 10029, USA

THE AIM of this study was to investigate the regulation of glial cell line-derived neurotrophic factor (GDNF) mRNA by activation of glutamate receptors in the rat striatum. We observed an increase in GDNF mRNA levels in the adult rat striatum after administration of subseizure doses of *N*-methyl-D,L-aspartic acid (NMA) and kainic acid. Since it is unclear whether the upregulation of GDNF occurred in neurons or astrocytes within the striatum, we further investigated whether GDNF gene expression in primary striatal astrocytes in culture could be regulated by glutamate receptor activation. We found that treatment of the cultures with NMA and kainic acid similarly upregulated GDNF gene expression as observed *in vivo*, suggesting that striatal astrocytes express functional glutamate receptors. Immunocytochemical and nuclease protection analysis revealed that striatal astrocytes expressed the NMDA-R1 subunit. These findings suggest the regulation of GDNF mRNA in the striatum may be mediated by excitation of glutamate receptors via glutamatergic cortical affertents.

Key words: Glial cell line-derived neurotrophic factor (GDNF); *N*-methyl-D,L-aspartic acid (NMA); Kainic acid; Striatum; Striatal astrocytes; NMDA receptor

[CA]Corresponding Author

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a potent neurotrophic factor that enhances the survival of dopaminergic neurons and motor neurons.[1,2] It is distantly related to the transforming growth factor-β superfamily.[1] In embryonic mesencephalic cultures, GDNF has been shown to promote survival and differentiation of dopamine neurons.[1] More recently, administration of GDNF was found to exert a protective and regenerative effect on the nigrostriatal dopamine system in MPTP-injected adult mice.[3] It has been suggested that GDNF might act as a typical target-derived neurotrophic factor in the nigro-striatal system.[4] Although GDNF mRNA was initially detected in developing but not in adult striatum,[5] we have recently been able to detect GDNF mRNA expression in the adult mouse striatum.[6] Other studies have shown GDNF mRNA to be expressed in the striatum of adult rats treated with a pilocarpine-induced epilepticus drug, suggesting the regulation of GDNF mRNA could be modified by excitatory cortical inputs.[4] Additional studies have also shown GDNF mRNA to be regulated by glutamate-mediated excitation in the hippocampus after kainic acid-induced seizures.[7]

In the present study, our aim was to investigate changes in GDNF mRNA levels upon activation of glutamate receptors in the striatum. Initially, we treated adult rats with the glutamate agonists NMA and kainic acid at subseizure levels in order to characterize the effects of these substances in the striatum *in vivo*. We further determined whether these glutamatergic effects on GDNF gene expression also occurred in striatal astrocytes *in vitro*. Such regulation would imply that striatal astrocytes have functional glutamate receptors, and we confirmed this using immunocytochemistry and nuclease protection assay for NMDA receptors *in vitro*.

Methods

In vivo study: Adult male Sprague–Dawley rats (250–300 g) were anesthetized with ketamine (50 mg kg^{-1}, i.m.) and xylazine (5 mg kg^{-1}, i.m.), and a silastic catheter was implanted into the right atrium through the jugular vein as previously described.[8] *N*-methyl-D,L-aspartic acid (14 mg kg^{-1}), kainic acid (2 mg kg^{-1}) or saline were injected i.v. once hourly for a total of 4 h and rats were killed 30 min after the last injection. RNA was isolated from striatum and was quantitated by a nuclease protection assay as described previously.[9]

In vitro study–primary striatal astrocyte culture: Postnatal day 3–4 Sprague–Dawley rats were decapitated, the striatum was dissected and meninges were removed. The tissue was trypsinized and DNase

treated for 15 min at 37°C, followed by a series of washes and centrifugations in MEM/Ham's F-12 supplemented with 10% fetal bovine serum. The resulting homogenate (cell suspension) was filtered through a sterile 37 μm nylon mesh screen. Cells were plated at ~ 1×10^6 cells per 60 mm dish in poly-L-lysine-coated culture dishes. The cultures were incubated at 37°C in an atmosphere of 8% CO_2, and 95% air.

Treatment: Cells at 5–7 days *in vitro* (> 90% confluent) were placed into defined medium for 24 h and then treated with NMA (50 μM), kainic acid (50 μM) or vehicle (100 mM sodium bicarbonate pH 8.0) for 0, 2, 4, 8 or 24 h ($n = 5$ for each group). RNA was isolated, and was quantitated by a nuclease protection assay as described previously.[9]

Immunocytochemistry: Astrocyte cultures were fixed with 4% paraformaldehyde plus 4% sucrose in PBS at 37°C for 40 min. All washes were with phosphate-buffered saline (PBS 0.1 M; pH 7.2) and all antibody incubations were diluted in their appropriate blocking solution as described below at room temperature. Cells that were to be stained with anti-NMDA-R1 were treated with 10% DMSO for 1 h, followed by a freeze thaw cycle to permeabilize the membrane. Cells were pre-incubated with 10% goat serum in PBS for 30 min and incubated with a mouse monoclonal anti-NMDA-R1 antibody (NMDA-R1 antibody 54.1, kindly provided by Dr J.H. Morrison, Mount Sinai School of Medicine)[10] at a dilution of 1:1000 overnight. Cells stained with anti-GFAP were pre-incubated with 10% goat serum, 0.3% triton in PBS for 30 min and incubated with the mouse monoclonal anti-GFAP antibody (Boehringer Mannheim) at a dilution of 1:50 overnight. Cells were then incubated with biotinylated anti-mouse IgG (Vector) at 1:200 for 2 h, followed by streptavidin–rhodamine (Molecular Probes) at 1:500 for 1 h. 4′,6-diamidino-2-phenylindole (DAPI) was used to visualize nuclear DNA staining (Sigma) at 1:1500 for 10 min. For immunocytochemical control, cultures were incubated in the absence of primary antibody.

Statistics: Significant differences in GDNF mRNA levels between control, kainic acid and NMA treatment groups were analyzed using analysis of variance (ANOVA) followed by Fisher's protected least significant difference *post hoc* analysis. The level of significance was set at $p < 0.05$.

Results

In our *in vivo* study, GDNF gene expression in the striatum was regulated by NMA and kainic acid. The dosages of NMA and kainic acid injected did not produce seizure activity nor, based on the results of others, were they likely to cause neurodegeneration. GDNF mRNA levels quantitated by a nuclease protection assay were significantly increased by kainic acid (2.3-fold, $p < 0.01$) and NMA (2.4-fold, $p < 0.006$) within 4 h compared with control saline-injected animals (Fig. 1A).

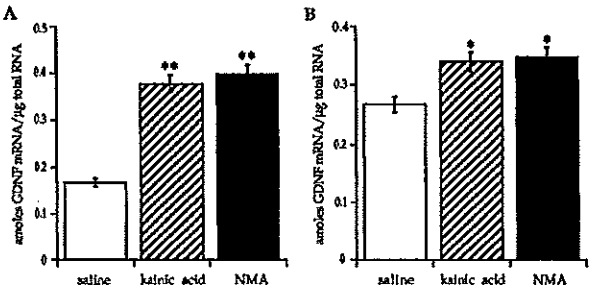


FIG. 1. Quantitative analysis of GDNF mRNA levels in the striatum after kainic acid and NMA treatment for 4 h. (A) *In vivo* study: GDNF mRNA levels increased significantly following kainic acid (2.3 fold, $p < 0.01$) and NMA (2.4 fold, p, 0.006) within 4 h compared with control saline-injected animals. (B) Primary striatal astrocyte cultures: GDNF mRNA increased significantly by kainic acid (1.3 fold $p < 0.04$) and NMA (1.3 fold, $p < 0.025$) within 4 h compared with control. Bar represents the mean ± s.e.m. for $n = 4$ *in vivo* and $n = 5$ *in vitro*.

To investigate whether the regulation of GDNF gene expression can be directly regulated in striatal astrocytes by glutamate receptor activation, primary striatal astrocyte cultures were treated with kainic acid, NMA or vehicle. In accordance with our

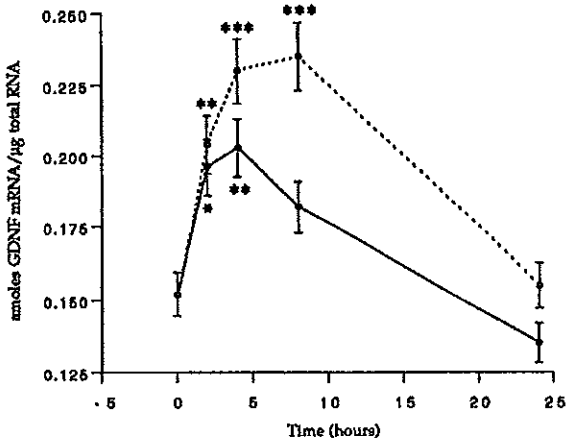
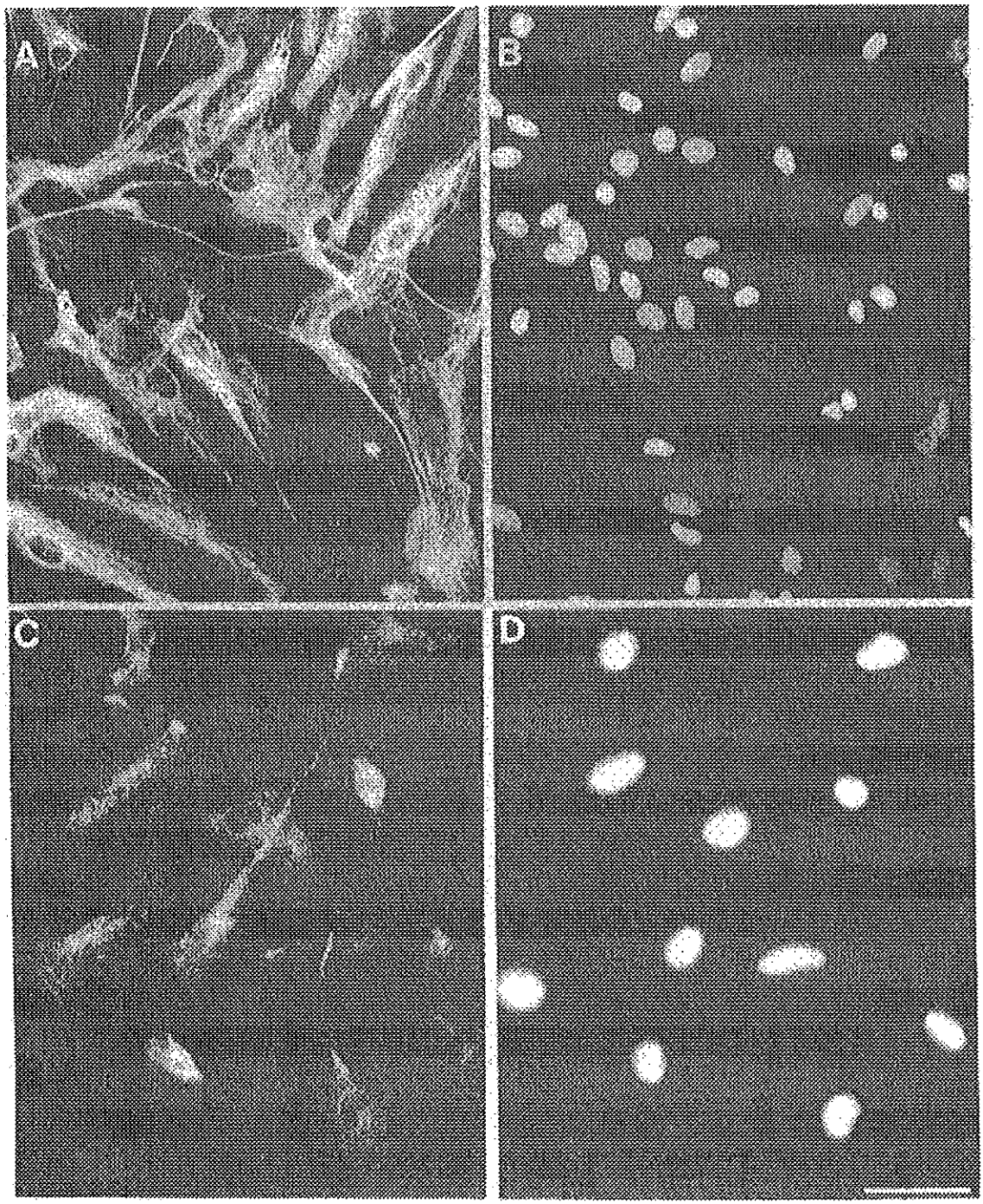


FIG. 2. Time-course quantitative analysis of GDNF mRNA levels in striatal astrocyte cultures 0, 2, 4, 8 and 24 h after kainic acid and NMA treatment. Kainic acid (dotted line) increased GDNF mRNA levels significantly by 2 h (34%, $p < 0.008$) and 4 h (51%, $p < 0.0002$) and was maximally increased at 8 h (64%, $p < 0.0001$). By 24 h, GDNF mRNA levels returned to control levels. NMA (solid line) also increased GDNF mRNA levels by 2 h (30%, $p < 0.05$) and 4 h (34%, $p < 0.01$), and by 8 and 24 h, GDNF mRNA levels decreased toward control levels. Each bar represents the mean ± s.e.m. for $n = 5$ for each group.


A
B
C
D
E
NMDA-R1 RNA STANDARDS
RNA
0 0.5 1 2 4 6 8 10

increase *in vivo*, we were also able to see an increase in GDNF mRNA at 4 h *in vitro* (Fig. 1B). We further characterized this effect using a time-course analysis in the primary striatal astrocyte cultures treated with kainic acid, NMA or vehicle. Kainic acid induced a significant 34% increase in GDNF mRNA levels by 2 h ($p < 0.008$), a 51% increase at 4 h ($p < 0.0002$) and was maximally induced by 54% compared to control at 8 h ($p < 0.0001$). By 24 h, GDNF mRNA levels returned to control levels (Fig. 2). NMA induced a significant 30% increase in GDNF mRNA levels by 2 h ($p < 0.05$) and a 34% increase at 4 h ($p < 0.01$). By 8 and 24 h, GDNF mRNA levels decreased toward control levels (Fig. 2).

The regulation of GDNF gene expression in the striatal astrocyte cultures by the application of NMA strongly suggests that astrocytes express NMDA receptors. Immunocytochemical staining of the primary striatal astrocyte cultures (5–7 days *in vitro*) demonstrated that > 90% of the cells are GFAP positive (Fig. 3A, B). When sister cultures were immunostained with a mouse anti-NMDA-R1 monoclonal antibody, most of the cells (> 90%) were found to be immunopositive, but there were considerable variations in the intensity of staining (Fig. 3C, D).

To support the presence of NMDA receptor expression in the striatal astrocyte culture, we determined whether NMDA-R1 mRNA could be detected by nuclease protection assay from these cultures and as shown in Fig. 3E, we were able to detect NMDA-R1 mRNA, albeit at low levels, in RNA extracted from these cultures.

Discussion

The present data demonstrate that GDNF mRNA is regulated by activation of glutamate receptors in the striatum. Several studies have demonstrated that growth factor gene expression can be regulated by excitatory amino acids.[11,12] It has been reported previously that GDNF mRNA levels are upregulated by kainic acid-induced seizures in the hippocampus.[7] In the *in vivo* study, we observed an increase in GDNF mRNA expression in the striatum in response to sub-seizure dosages of NMA and kainic acid after 4 h of treatment. Our finding that exogenous administration of glutamate agonists stimulates GDNF gene expression suggests that endogenous glutamatergic inputs regulate GDNF mRNA levels under physiological conditions. Thus, in context of known basal ganglia circuitry whereby the striatum receives major excitatory glutamatergic afferents from the cortex, our data suggest that GDNF gene expression may be regulated by these cortical inputs. This observation is also consistent with the finding that GDNF mRNA levels are upregulated by pilocarpine-induced seizures, which activate the cortical-striatal pathway.[4]

Our *in vivo* data clearly indicate that GDNF is synthesized in the adult rat striatum. Although it has been reported that GDNF mRNA is not expressed in the striatum of adult rats by *in situ* hybridization,[5,13] we were able to detect GDNF mRNA using the more sensitive nuclease protection assay. However, the cellular localization of where GDNF is synthesized *in vivo* is still yet to be elucidated. While GDNF was originally isolated from a glial cell line, it has been suggested that GDNF may be expressed in neurons[4] or in astrocytes[5] in the striatum. Recent studies have reported cellular expression of GDNF mRNA in substantia nigra *in vivo* and in basal forebrain Type 1 (T1) astrocytes *in vitro* from developing rats using PCR analysis.[14] The detection of GDNF mRNA by a nuclease protection assay in RNA isolated from primary striatal astrocyte cultures in the present study demonstrates that GDNF can also be synthesized by striatal astrocytes, at least *in vitro*.

Several studies have demonstrated functional neurotransmitter receptors on astrocytes which, when stimulated, cause an elevation of oscillatory cytoplasmic free calcium, which in turn functions to regulate signalling pathways within neurons.[15] To investigate whether GDNF gene expression can be directly regulated by neurotransmitter receptor activation on astrocyte derived from the striatum, primary striatal astrocytes cultures were treated with NMA, kainic acid or vehicle. Our *in vitro* studies revealed a rapid increase in GDNF mRNA levels within 2 and 4 h after treatment with both glutamate agonists. These results suggest that GDNF mRNA may also be directly regulated in astrocytes in the striatum *in vitro*.

The regulation of GDNF gene expression by NMA in striatal astrocytes *in vitro* strongly indicates the presence of functional NMDA receptors on astrocytes. Although there is extensive evidence for the presence of kainate and AMPA receptors on astrocytes,[16] there is little evidence for functional NMDA

FIG. 3. Immunocytochemical and nuclease protection analysis of the NMDA-R1 subunit in striatal astrocyte cultures. (A) GFAP staining of primary striatal astrocyte cultures at 5–7 days *in vitro*, > 90% stained positive for GFAP in relation to DAPI nuclear staining shown in (B). (C) Sister cultures were immunostained with mouse anti-NMDA-R1 monoclonal antibody, > 90% were found to be immunopositive in relation to DAPI nuclear staining shown in panel (D). Bar = 50 μm (A,B), 30 μm (C,D). (E) Nuclease protection assay of NMDA-R1 mRNA in striatal astrocyte cultures. A standard curve with increasing amounts (0–10 μl of a 100 fg μl^{-1} NMDA-R1 (+) strand) of reference RNA is shown on the left, and a sample of RNA (76 μg) extracted from the striatal astrocyte cultures expressing NMDA-R1 mRNA (0.062 amol μg^{-1} total RNA) is shown on the right.

receptors on astrocytes. Recently, however, evidence of NMDA-R1 immunoreactivity has been localized to astrocytes in the visual cortex of adult and neonatal rats.[17] In addition, NMDA receptors have been proposed to exist on another population of astrocytes, the retinal glial cells, and activation of NMDA receptor has been demonstrated to stimulate their proliferation.[18] Electrophysiologic studies showed Bergmann glial cells in the cerebellum to have a distinct intracellular response to NMDA.[19] In our striatal astrocyte cultures, we have demonstrated the presence of the NMDA-R1 subunit of the NMDA receptor by immunocytochemistry. In agreement with our immunocytochemical finding, we were able to detect NMDA-R1 mRNA by nuclease protection assay.

Conclusion

The present results demonstrate that GDNF mRNA levels in the striatum are upregulated by activation of glutamate receptors. Our *in vivo* study showed an increase in GDNF mRNA levels in adult rat striatum after NMA and kainic acid treatments. Furthermore, we found an increase in GDNF mRNA levels in primary striatal astrocyte cultures after glutamate agonist treatment. Lastly, we provided evidence for functional NMDA receptors on striatal astrocytes *in vitro*. It has been suggested by other studies that GDNF might act as a typical target-derived neurotrophic factor for mesencephalic dopamine neurons.[3] Our data support the concept that GDNF gene expression is regulated by glutamatergic cortical afferents. This present study indicates that GDNF can be transsynaptically regulated by glutamatergic cortico-striatal inputs which regulate the availability of this dopaminergic trophic factor which in turn may be retrogradely transported to dopamine neurons.

References

1. Lin L-F H, Doherty DH, Lile JD *et al. Science* 260, 1130–1132 (1993).
2. Henderson CE, Phillips HS, Pollock RA *et al. Science* 266, 1062–1064 (1994).
3. Tomac A, Lindqvist E, Lin L-F H *et al. Nature* 373, 335–339 (1995).
4. Schmidt-Kastner R, Tomac A, Hoffer B *et al. Mol Brain Res* 26, 325–330 (1994).
5. Stromberg I, Bjorklund L, Johansson M *et al. Exp Neurol* 124, 401–412 (1993).
6. Blum M and Weickert CS. *Neurobiol Aging* (in press) (1995).
7. Humpel C, Hoffer B, Stromberg I *et al. Neurosci* 59, 791–795 (1994).
8. Gore AC and Roberts JL. *Endocrinology* 134, 2026–2031 (1994).
9. Blum M. *Methds Enzymol* 168, 618–633 (1989).
10. Siegel SJ, Brose N, Janssen WG *et al. Proc Natl Acad Sci USA* 91, 564–568 (1994).
11. Mattson MP, Murrain M, Guthrie PB *et al. J Neurosci* 9, 3728–3740 (1989).
12. Zafra F, Hengerer B, Leibrock J *et al. EMBO J* 9, 3545–3550 (1990).
13. Poulsen KT, Armanini MP, Klein RD *et al. Neuron* 13, 1245–1252 (1994).
14. Schaar DG, Sieber B-A, Dreyfus CF *et al. Exp Neurol* 124, 368–371 (1993).
15. Cornell-Bell AH, Finkbeiner SM, Cooper MS *et al. Science* 247, 470–473 (1990).
16. McNaughton LA and Hunt SP. *Mol Brain Res* 16, 261–268 (1992).
17. Aoki C, Venkatesan C, Go C-G *et al. J Neurosci* 14, 5202–5222 (1994).
18. Uchihori Y and Puro DG. *Brain Res* 613, 212–220 (1993).
19. Muller T, Grosche J, Ohlemeyer C *et al. NeuroReport* 4, 671–673 (1993).

ACKNOWLEDGEMENTS: This work was supported by NIH grant AG-08538 and an Irma T. Hirschl Career Scientist Award given to M.B. We would like to thank Dr Deanna Benson for her helpful advice and contribution to the NMDA-R1 subtype receptor immunocytochemistry analysis.

Received 13 March 1995; accepted 28 April 1995