Endocrine, *vol. 6, no. 1, 1–3, February 1997* 0969-711X/97/6:1-3/$8.75

REVIEW

Commentary on the Use of Immortalized Neuroendocrine Cell Lines for Physiological Research

Michael Selmanoff

Department of Physiology, University of Maryland, School of Medicine, Baltimore, MD

A great deal of work has been done recently using the GT_{1-7} immortalized cells *(1)* as a model of a "highly differentiated" GnRH neuron (reviewed in refs. *2–5*). GT_1 cells, maintained in cell culture, exhibit several morphological *(1,6)*, biochemical *(1,7)*, and physiological *(1,3)* functions characteristic of adult, differentiated GnRH neurons. In addition, though never demonstrated for GnRH neurons *in situ*, GT_1 cells in culture exhibit intrinsic, pulsatile secretory activity *(8–10)*. They have been found to "differentiate" *in situ* and restore reproductive function in adult *hpg* mice following their direct introduction into the brain *(11)*. Whereas several advantages of using GT_1 cells to model the GnRH neuron have been enumerated *(2–4)*, potential disadvantages have received less attention. The in vitro study of such transformed cell lines has several limitations. The extent to which observed electrophysiological, receptor, second messenger, gene expression, and secretory properties of GT_1 cells are subsequently found accurately to characterize *in situ* GnRH neurons in adult brain is unknown. Potential limitations include the following problems, several of which are common to the study of all cell lines.

First, it is unclear whether the level of differentiation of postmitotic, immortalized cells can be maintained over cell generations. For instance, increased expression of T-antigen in transformed pituitary lactotroph cell lines is correlated with progressive loss of the lactotroph phenotype *(12)*. If this occurs with increasing numbers of cell passages, to what extent is the fully differentiated phenotype compromised by the lesser concentrations of T-antigen found in GT_1 founder cells? Second, significant effects of T-antigen expression in these cells on normal physiological cell functions may exist, but are unrecognized. Third, when stereotaxically placed in the hypothalamus of *hpg* mice, axons derived from normal GnRH cells usually innervate the median eminence *(13,14)* whereas GT_1-derived axons seldom do so *(11)*. Such GT_1 hypothalamic grafts, of course, exhibit a marked propensity to form tumors *(11)*. Fourth, studied in vitro, a homogeneous cell line is obviously removed from normal in situ interactions with other afferent neurons and supporting glia. Fifth, several million GT_1 cells are commonly studied on coverslips or in culture wells whereas the rodent neuroendocrine brain has about 800 GnRH neurons *(15,16)*, which likely exhibit considerably less GnRH-GnRH connectivity than the in vitro cultures. Sixth, GT_1 cells express neurotransmitter receptors (e.g., β_1-adrenergic) and respond to the corresponding ligands (e.g., isoproterenol), in cases for which there is considerable evidence against *in situ*. For example, substantial evidence exists for α- and not β-adrenergic receptor mediation in vivo *(17–21)*. Another example is the $GABA_A\alpha_1$ subunit that is reportedly expressed in GT_1 cells *(22,23)*, but not by GnRH neurons *in situ (24,25)*. Similarly, *in situ*, about 30% of GnRH neurons are observed to express the $GABA_A\gamma_2$ receptor subtype (Dr. Allan Herbison, personal communication), whereas three groups have reported an absence of γ subunit mRNAs (γ_1, γ_2, γ_{2L}, and γ_3) from GT_1 cells determined by Northern blot analysis and RT-PCR *(22,23,26)*. Accordingly, GT_1 cells *(22,23)* lack the γ subunit-associated sensitivities to benzodiazepines *(27–29)*, ethanol *(30)*, and Zn^{2+} *(31)*. Finally, particularly disturbing is recent evidence that GT_1 cells exhibit several properties of "embryonic," not adult GnRH neurons. It has been known for some time that immortalized cell lines usually represent or are "fixed" at a particular stage of cellular development, that correct developmental targeting (e.g., to the fully differentiated, adult phenotype) in transgenic animals is at present quite problematic, and that, in fact, most such cell lines produced are "immature" neurons *(32,33)*. Five groups now have reported that GABA and GABA agonists, acting via $GABA_A$ receptors, stimulate GnRH release from GT_1 cells *(23,26,34–36)*. In adult brain, a direct, stimulatory GABA action is extremely rare and limited in circumstance, both

Received September 16, 1996; Revised November 14, 1996; Accepted November 14, 1996.

Author to whom all correspondence and reprint requests should be addressed: Dr. Michael Selmanoff, Department of Physiology, University of Maryland, School of Medicine, 655 West Baltimore Street, Baltimore, MD 21201-1559. E-mail: mks@umabnet.ab.umd.edu

in the neuroendocrine and in the general neurobiological literature (*see*, e.g., refs. *37–39*). GABA is thought to be the most ubiquitous inhibitory neurotransmitter in brain *(40,41)*, with about 33% of the estimated 10^{15} CNS synapses utilizing GABA as their transmitter *(42)*, and virtually every neuron in adult brain studied to date is inhibited by applied GABA *(43)*. However, *in utero* and during early postnatal life, there is now substantial evidence that GABA acts as an excitatory neurotransmitter causing $GABA_A$-mediated depolarizing potentials, perhaps mediated by L-type Ca^{2+} channel activation (*see*, e.g., refs. *44–53*). GABA then reverses its action and starts to play its well-known role as an inhibitory neurotransmitter in adult brain (for reviews, *see* refs. *54–56*). Indeed, GT_1 cells share several intrinsic properties such as voltage- and ligand-gated ion channels, resting membrane potential, and receptors expressed, with "embryonic" GnRH neurons studied in organotypic cell culture, including GABA-induced depolarization via $GABA_A$ receptors *(57)*. Their "embryonic" nature may in fact be essential for their successful integration into the adult *hpg* mouse hypothalamus *(11)*. Work with the human GnRH promoter-SV40 T-antigen oncogene fusion gene construct (the rat GnRH promoter was used to generate the GT_1 cells) in transgenic animals clearly indicates that T-antigen expression begins, and presumably, the cells are transformed, during embryogenesis as they migrate from the olfactory placode to the rostral hypothalamus *(58,59)*.

Given this substantive evidence, it would appear that further study of GT_1 cells, and GnRH neurons in organotypic cell cultures, for purposes other than modeling the late embryonic/early postnatal developmental period, may be misguided. As a model for studying adult GnRH pulse generator function, I agree with Kusano, Fueshko, Gainer, and Wray *(57)* who recently concluded: "in other neuronal systems, it has been shown that novel oscillatory rhythms and synchronous firing in populations of cells can emerge as a result of the interaction of the intrinsic neuronal biophysical properties and their resonance with specific neuronal inputs...it is likely that a full mechanistic description of GnRH secretory rhythms in vivo will require such network analyses." Another issue regards in vivo vs in vitro gene promoter analysis. Considering heterologous cell systems, Dutt, Kaplitt, Kow, and Pfaff *(60)* have argued: "considering that a high proportion of functional studies of promoters are carried out in arbitrarily chosen cell lines whose nuclear protein environment may be more or less irrelevant to the normal nerve cell...it is desirable...to determine...functional importance...in...neurons which have normal cell-to-cell contacts." Indeed, progress continues to be made on in vivo promoter analysis of neuroendocrine genes (*see*, e.g., refs. *61–63*), and the related capability for ex vivo transfection of gene promoter-reporter constructs into identified neurons in brain slices using biolistics and immunohistochemistry (*see*, e.g., refs. *64–67*). Although not a heterologous cell type, the GT_1 cells are not, strictly speaking, a homologous system either, because they are transformed GnRH cells. More importantly, if the concentrations of transcription factors (enhancers and or repressors), known to be critical for gene promoter function, vary with development, then GnRH gene promoter analyses carried out in GT_1 cells may yield results at variance with the adult, fully differentiated GnRH neuron studied *in situ*. A related problem could arise if T-antigen expression increases with time and or successive cell passages. This would result in increased copies of T-antigen-linked GnRH promoter available to bind transcription factors. A transcription factor present in limited concentration could be, in effect, "quenched" by the introduced transgene, resulting in a markedly decreased or absent signal from the reporter gene construct and an incorrect interpretation of the experimental result.

Investigators must remain appropriately vigilant for any/all potential limitations of genetically engineered cells and their "physiological" responses. In vivo work, including in vivo gene transfer for gene promoter studies, however challenging, still must be done. For the same reasons that clinical trials for drug approval are conducted in vivo in humans, the sine qua non for physiological relevance is work performed or validated in vivo. Molecular genetic work must be grounded in normal cellular and physiological contexts, otherwise some (perhaps much) of it will undoubtedly be found to be physiologically erroneous.

References

1. Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padula, C. A., Roberts, J. L., and Weiner, R. I. (1990). *Neuron* **5,** 1–10
2. Mellon, P. L., Wetsel, W. C., Windle, J. J., Valenca, M. M., Goldsmith, P. C., Whyte, D. B., Eraly, S. A., Negro-Vilar, A., and Weiner, R. I. (1992). *Ciba. Found. Symp.* **168,** 104–117.
3. Weiner, R. I., Westsel, W., Goldsmith, P., Martínez de la Escalera, G., Windle, J., Padula, C., Choi, A., Negro-Vilar, A., and Mellon, P. (1992). *Front Neuroendocrinol.* **13,** 95–119.
4. Lawson, M. A., Whyte, D. B., Eraly, S. A., and Mellon, P. L. (1995). Rec. Prog. Horm. Res. **50,** 459–463.
5. Sagrillo, C. A., Grattan, D. R., McCarthy, M. M., and Selmanoff, M. (1996). *Behav. Genet.* **26,** 241–277.
6. Liposits, Z., Merchenthaler, I., Wetsel, W. C., Reid, J. J., Mellon, P. L., Weiner, R. I., and Negro Vilar, A. (1991). *Endocrinology* **129,** 1575–1583.
7. Wetsel, W. C., Mellon, P. L., Weiner, R. I., and Negro-Vilar, A. (1991). *Endocrinology* **129,** 1584–1595.
8. Martínez de la Escalera, G., Choi, A. L. H., and Weiner, R. I. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 1852–1855.
9. Wetsel, W. C., Valenca, M. M., Merchenthaler, I., Liposits, Z., Lopez, F. J., Weiner, R. I., Mellon, P. L., and Negro-Vilar, A. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 4149–4153.
10. Krsmanovic, L. Z., Stojilkovic, S. S., Merelli, F., Dufour, S. M., Virmani, M. A., and Catt, K. J. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 3908–3912.
11. Silverman, A.-J., Roberts, J. L., Dong, K.-W., Miller, G. M., and Gibson, M. J. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 10,668–10,672.
12. Mellon, P. L., Windle, J. J., and Weiner, R. I. (1991). *Rec. Prog. Horm. Res.* **47,** 69–93.

13. Silverman, A.-J., Zimmerman, E. A., Gibson, M. J., Perlow, M. J., Charlton, H. M., and Krieger, D. T. (1985). *Neuroscience* **16,** 69–84.
14. Miller, G. M., Silverman, A.-J., Rogers, M.-C., and Gibson, M. J. (1995). *Biol. Reprod.* **52,** 572–583.
15. Wray, S. and Hoffman, G. (1986). *J. Comp. Neurol.* **252,** 522–531.
16. Wray, S. and Gainer, H. (1987). *Neuroendocrinology* **45,** 413–419.
17. Weiner, R. I. and Ganong, W. F. (1978). *Physiol. Rev.* **58,** 905–976.
18. Barraclough, C. A., Wise, P. M., and Selmanoff, M. K. (1984). *Rec. Prog. Horm. Res.* **40,** 486–529.
19. Ramirez, V. D., Feder, H. H., and Sawyer, C. H. (1984). In: *Frontiers in Neuroendocrinology*. Martini, L. and Ganong, W. F. (eds.). Raven: NY, pp. 27–84.
20. Kordon, C., Drouva, S. V., Martínez de le Escalera, G., and Weiner, R. I. (1994). In: *The Physiology of Reproduction.* 2nd ed. Knobil, E., Neill, J. D., et al. (eds.). Raven: NY, pp. 1621–1682.
21. Terasawa, E. (1995). *Cell. Mol. Neurobiol.* **15,** 141–164.
22. Hales, T. G., Kim, H., Longoni, B., Olsen, R. W., and Tobin, A. J. (1992). *Mol. Pharmacol.* **842,** 197–202.
23. El-Etr, M., Akwa, Y., Fiddes, R. J., Robel, P., and Baulieu, E.-E. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 3769–3773.
24. Petersen, S. L., McCrone, S., Coy, D., Adelman, J. P., and Mahan, L. C. (1993). *Endocr. J.* **1,** 29–34.
25. Petersen, S. L. and McCrone, S. (1994). In: In Situ *Hybridization Histochemistry in Neurobiology, Advances in Methodology.* Eberwine, J. H., Valentino, K. L., and Barchas, J. D. (eds.). Oxford: NY, pp. 78–95.
26. Favit, A., Wetsel, W. C., and Negro-Vilar, A. (1993). *Endocrinology* **133,** 1983–1989.
27. Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, P., Schofield, P. R., and Seeburg, P. H. (1989). *Nature* **338,** 582–585.
28. Ymer, S., Draguhn, A., Wisden, W., Werner, P., Keinänen, K., Schofield, P. R., Sprengel, R., Pritchett, D. B., and Seeburg, P. H. (1990). *EMBO J.* **9,** 3261–3267.
29. Knoflach, F., Rhyner, T., Villa, M., Kellenberger, S., Drescher, U., Malherbe, P., Sigel, E., and Möhler, H. (1991). *FEBS Lett.* **293,** 191–194.
30. Wafford, K. A., Burnett, D. M., Leidenheimer, N. J., Burt, D. R., Wang, J. B., Kofuji, P., Dunwiddie, T. V., Harris, R. A., and Sikela, J. M. (1991). *Neuron* **7,** 27–33.
31. Draguhn, A., Verdorn, T. A., Ewert, M., Seeburg, P. H., and Sakmann, B. (1990). *Neuron* **5,** 781–788.
32. Rosenfeld, M. G., Crenshaw, E. B. III, and Lira, S. A. (1988). *Ann. Rev. Neurosci.* **11,** 353–372.
33. Cepko, C. L. (1989). *Ann. Rev. Neurosci.* **12,** 47–65.
34. Hales, T. G., Sanderson, M. J., and Charles, A. C. (1994). *Neuroendocrinology* **59,** 297–308.
35. Martínez de la Escalera, G., Choi, A. L. H., and Weiner, R. I. (1994). *Neuroendocrinology* **59,** 420–425.
36. Spergel, D. J., Krsmanovic, L. Z., Stojilkovic, S. S., and Catt, K. J. (1995). *Neuroendocrinology* **61,** 499–508.
37. Alger, B. E. and Nicoll, R. A. (1982). *J. Physiol.* **328,** 125–141.
38. Alkon, D. L., Sanchez-Andres, J.-V., Ito, E., Oka, K., Yoshioka, T., and Collin, C. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 11,862–11,866.
39. Smith, R. L., Clayton, G. H., Wilcox, C. L., Escudero, K. W., and Staley, K. J. (1995). *J. Neurosci.* **15,** 4057–4067.
40. Roberts, E. and Sherman, M. A. (1993). *Neurochem. Res.* **18,** 365–376.
41. Roberts, E. (1995). *Biochem. Pharmacol.* **49,** 1–16.
42. Mugnaini, E. and Oertel, W. H. (1985). *Handbk. Chem. Neuroanat.* **4,** 436–608.
43. Roberts, E. (1991). *Neurochem. Res.* **16,** 409–421.
44. Mueller, A. L., Taube, J. S., and Schwartzdroin, P. A. (1984). *J. Neurosci.* **4,** 860–867.
45. Janigro, D. and Schwartzdroin, P. A. (1988). *Brain Res.* **469,** 171–184.
46. Ben-Ari, Y., Cherubini, E., Corradetti, R., and Gaiarsa, J. L. (1989). *J. Physiol.* **416,** 303–325.
47. Swann, J. W., Brady, R. J., and Martin, D. L. (1989). *Neuroscience* **28,** 551–561.
48. Luhmann, H. J. and Prince, D. A. (1991). *J. Neurophysiol.* **65,** 247–263.
49. Leinekugel, X., Tseeb, V., Ben-Ari, Y., amd Bregestovski, P. (1995). *J. Physiol.* **487,** 319–329.
50. LoTurco, J. J., Owens, D. F., Heath, M. J. S., Davis, M. B. E., and Kriegstein, A. R. (1995). *Neuron* **15,** 1287–1298.
51. Obrietan, K. and van den Pol, A. N. (1995). *J. Neurosci.* **15,** 5065–5077.
52. Obrietan, K. and van den Pol, A. N. (1996). *J. Neurosci.* **16,** 3521–3533.
53. Chen, G., Trombley, P. Q., and van den Pol, A. N. (1996). *J. Physiol.* **494,** 451–464.
54. Cherubini, E., Gaiarsa, J. L., and Ben-Ari, Y. (1991). *Trends Neurosci.* **14,** 515–519.
55. Ben-Ari, Y., Tseeb, V., Raggozzino, D., Khazipov, R., and Gaiarsa, J. L. (1994). *Prog. Brain Res.* **102,** 261–273.
56. Gaiarsa, J. L., McLean, H., Congar, P., Leinekugel, X., Khazipov, R., Tseeb, V., Ben-Ari, Y. (1995). *J. Neurobiol.* **26,** 339–349.
57. Kusano, K., Fueshko, S., Gainer, H., and Wray, S. (1995). *Proc. Natl. Acad. Sci. USA* **92,** 3918–3922.
58. Radovick, S., Wray, S., Lee, E., Nichols, D. K., Nakayama, Y., Weintraub, B. D., Westphal, H., Cutler, G. B. Jr., and Wondisford, F. E. (1991). *Proc. Natl. Acad. Sci. USA* **88,** 3402–3406.
59. Wolfe, A. M., Wray, S., Westphal, H., and Radovick, S. (1996). *J. Biol. Chem.* **271,** 20,018–20,023.
60. Dutt, A., Kaplitt, M. G., Kow, L.-M., and Pfaff, D. W. (1994). *Neuroendocrinology* **59,** 413–419.
61. Kaplitt, M. G., Rabkin, S. D., and Pfaff, D. W. (1993). *Curr. Topics Neuroendocrinol.* **11,** 169–191.
62. Radovick, S., Wray, S., Muglia, L., Westphal, H., Olsen, B., Smith, E., Patriquin, E., and Wondisford, F. E. (1994). *Horm. Behav.* **28,** 520–529.
63. Kaplitt, M. G., Kwong, A. D., Kleopoulos, S. P., Mobbs, C. V., Rabkin, S. D., and Pfaff, D. W. (1995). *Proc. Natl. Acad. Sci. USA* **91,** 8979–8983.
64. Johnston, S. A. (1990). *Nature* **346,** 776,777.
65. Cheng, L., Ziegelhoffer, P. R., and Yang, N. S. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 4455–4459.
66. Jiao, S., Cheng, L., Wolff, J. A., and Yang, N. S. (1993). *Biotechnology* **11,** 497–502.
67. Lo, D. C., McAllister, A. K., and Katz, L. C. (1994). *Neuron* **13,** 1263–1268.