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Thyroid Parafollicular Cells

An Accessible Model for the Study of Serotonergic Neurons

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Abstract

Serotonergic neurons play key roles in modulating a wide variety of behavioral and homeostatic processes. However, there is a paucity of good model systems to study these neurons at a molecular level. In this review we will present evidence that cell lines derived from an unexpected source, thyroid parafollicular cells (PF) (also called C cells), fit the criteria for use as models for the study of serotonergic neurons. A strength of PF cell lines over other cell lines is that the parental PF cells have serotonergic properties and a neuronal potential that is consistent with their neural crest origin. Furthermore, PF cells and PF cell lines are capable of expressing the fundamental properties of serotonergic neurons, including: (1) serotonin (5-HT) biosynthesis by tryptophan hydroxylase (TPH), (2) vesicular 5-HT storage and regulated release, (3) expression of a 5-HT autoreceptor, and (4) expression of the 5-HT transporter. In this review, we will focus primarily on the serotonergic and neuronal properties of the rat CA77 PF cell line and the parental rat PF cells. The applicability of CA77 cells for molecular analyses will be described. First, their use for studies on the glucocorticoid regulation of the TPH gene will be discussed. Second, control of the calcitonin/calcitonin gene-related peptide (CT/CGRP) gene will be discussed, with particular emphasis on the application of serotonergic drugs in treating migraine headaches. These examples highlight the versatility of thyroid PF cell lines as a system for studying the control of both serotonin biosynthesis and physiological actions.

Index Entries: Parafollicular cell; C cell; serotonin; CGRP; calcitonin; sumatriptan; dexamethasone; neural crest; tryptophan hydroxylase; transcription.

Introduction

In this review, we will describe the relative advantages of a thyroid parafollicular (PF) cell line as model system for studying serotonergic neurons. It is, however, first prudent to ask the question: "why even use a cell line?" There is a partially justifiable prejudice against neuronal cell lines. Among the obvious drawbacks of cell lines are the lack of appropriate extracellular

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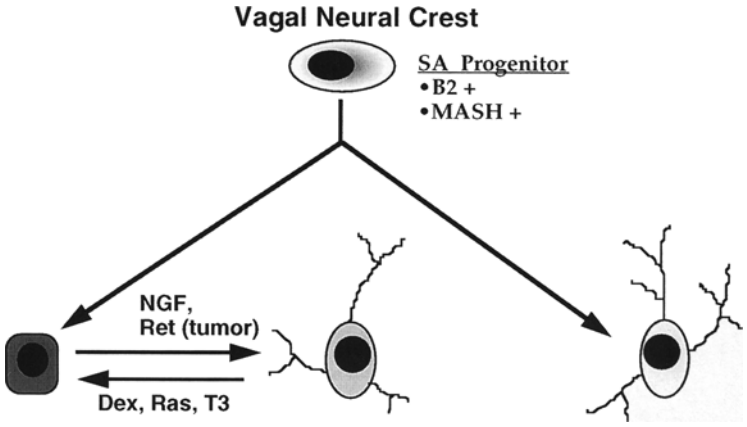
contacts and targets, as well as appropriate growth controls and developmental history. Nonetheless, a homogenous population of cells is necessary for many biochemical and molecular studies. The heterogeneity of the brain stands as a formidable barrier for investigations into molecular mechanism. Similarly, collection of nucleic acids or proteins from microdissected nerve tissue can be practically and financially daunting. Consequently, over the past decade a number of laboratories have expanded the repertoire of cell lines applicable to neurobiological questions. Within their obvious limits, the judicious use of cell lines has greatly facilitated advances in neurobiology (Lendahl and McKay, 1990; Russo and Green, 1995).

What criteria might we place on the selection of a cell line as a model for serotonergic neurons? Some fundamental requirements of such a model system are: (1) expression of a neuronal phenotype; (2) presence of the fundamental serotonergic properties of 5-HT synthesis and regulated release, 5-HT sensing, and 5-HT uptake; and (3) confidence that the neuronal and serotonergic features are not the result of ectopic gene expression. Many previous studies have used the platelet as a model system, because it can take up and store 5-HT (Da Prada and Pletscher, 1968), despite numerous differences between these cells and serotonergic neurons, as reviewed by Gershon and Tamir (1984). Other studies have used neuroblastoma cell lines (Henley and Nielson, 1994), which often have an uncertain origin and commonly exhibit ectopic gene expression. The problem of ectopic expression in tumor cell lines is subtle, but potentially damaging, since inappropriate expression of a single gene could occur in the absence of the intracellular signaling proteins and regulatory proteins that would exist in serotonergic neurons. Thus, a key argument in favor of PF cell lines is their relationship with the parental PF cell phenotype. All the serotonergic features seen with PF cell lines have now been documented in normal PF cells, either in viva or in primary cultures. Hence, the serotonergic and neuronal features of PF cell lines appear to be a result of ontogeny rather than a consequence of transformation. Finally, it must be noted that the PF cell system may have drawbacks for certain studies since its neural crest developmental history differs from neural tube-derived CNS serotonergic neurons, but is similar to the serotonergic enteric neurons of the gut. However, as noted by Gershon et al. (1994), the ultrastructure of the enteric nervous system resembles the CNS rather than the peripheral nervous system. Thus, differing ontogenies may not always constitute a confounding issue. Nonetheless, other cell lines are currently being developed that may overcome this potential problem should it occur. For example, Whittemore and colleagues have recently constructed a temperature-sensitive cell line derived from SV 40 immortalized embryonic raphe neurons (White et al., 1994). Hence, comparison of results obtained from PF cells with other serotonergic lines may help reveal the common and distinguishing features between central and peripheral serotonergic neurons.

Serotonergic Neuronal Features of PF Cells

Neural Crest Ontogeny

The foundation for using thyroid PF cells (also called C cells) as a model system for studying serotonergic neurons lies in their intrinsic serotonergic properties and neuronal potential. Yet PF cells are generally viewed as endocrine or neuroendocrine cells. How did PF cells and PF cell lines acquire these features if they are not neurons? The answer is apparent in their origin from the neural crest. The neural crest is a transitory structure during embryogenesis that gives rise to a wide variety of cell types, including PF cells (LeDouarin et al., 1974; Polak et al., 1974; LeDouarin, 1982). A hallmark of the neural crest is the plasticity of differentiation pathways that are determined to a large extent by the local microenvironment. Progenitor cells initially have multiple developmental potentials



	Thyroid PF cell	Cultured PF cells	Enteric Neuron
•B2	+	-	-
•MASH	+	+/-	-
•NF	-	+	+
•CT/CGRP	CT/cgrp	ct/CGRP	CGRP
•5-HT	+	+	+
•5-HT transporter	-	+	+
•5-HT 1B receptor	+	+	+
•SBP	+	+	+
•MAO	+	+	+
•Ret	nd	+	+

Fig. 1. Neural crest ontogeny and in vitro plasticity of thyroid PF cells. The sympathoadrenal progenitor in the vagal neural crest is proposed to give rise to either thyroid PF cells or enteric neurons, depending on the embryonic migration pathway. Primary cultures of PF cells can be induced to a more neuronal phenotype by NGF. Tumors of PF cells, in which the Ret receptor has been activated, also display a more neuronal phenotype. The neuronal differentiation of the cell lines can be partially repressed to favor a more endocrine state by the glucocorticoid dexamethasone (Dex) or expression of the Harvey *ras* oncogene, both of which act to slow cell growth. Thyroid hormone (T3) treatment can suppress some of the neuronal morphology acquired by primary cultures. At the bottom of the figure are listed some differentiation state markers that are present (+) or absent (–) from the indicated cell types. In the case of MASH-1, it is still expressed in the PF cell lines, but not in primary cultures. The CT/CGRP alternative RNA processing choice yields varying ratios of CT to CGRP mRNAs in different cell lines, but always shows an increase in the ratio of CGRP/CT relative to the parental PF cells. The relative CT and CGRP expression levels are indicated by placing the minor species in lower case. Ret expression in the thyroid PF cells has not been determined (nd). Abbreviations: B2, B2 cell surface antigen; MASH, mammalian achaete-scute homolog 1; NF, neurofilaments; CGRP, calcitonin gene-related peptide; CT, calcitonin; 5-HT, serotonin; SBP, serotonin-binding protein; MAO, monoamine oxidase.

that are progressively restricted during migration, yet can be maintained to limited degrees even in the adult animal. The classic example of this plasticity is the endocrine adrenal chromaffin cell, which can become neuronal-like in culture (Unsicker et al., 1978; Doupe et al., 1985; Anderson, 1993). Hence the ability of endocrine PF cells to become neuronal-like in culture has precedence in the neural crest.

The neuronal features of PF cells are most reminiscent of neural crest-derived enteric neurons. Both PF cells and enteric neurons arise from the vagal subdivision of the neural crest. The PF cell progenitors comigrate from the neural tube with the enteric neuroblasts into the branchial arch mesenchyme (LeDouarin, 1982; Ciment and Weston, 1983, 1985; Payette et al., 1984; Ito and Sieber-Blum, 1991, 1993). The similarities between enteric neurons and PF cells is extended beyond their shared origin and migration pathways (Fig. 1). Both enteric neurons (Baetge et al., 1990; Carnahan et al.,

1991; Blaugrund et al., 1996) and PF cells (Clark et al., 1995a) have been proposed to arise from a sympathoadrenal progenitor. The sympathoadrenal progenitor has been well documented as the precursor to sympathetic neurons and adrenal chromaffin cells (for review, *see* Anderson, 1993). Both enteric neurons and PF cells express the sympathoadrenal markers MASH-1 and B2 (Johnson et al., 1990; Carnahan et al., 1991; Clark et al., 1995a; Blaugrund et al., 1996). However, careful analyses of mice lacking the ret gene (discussed below) have revealed some unexpected complexity within the sympathoadrenal lineage. Based on lineage tracings and dependence on Ret for development, Durbec et al. (1996a) have proposed that the majority of enteric neurons arise from a unique neural crest lineage, which they have termed the sympathoenteric lineage. The sympathoenteric and sympathoadrenal lineages share phenotypic similarities between both the progenitors and final cellular phenotypes. It appears that the major difference lies in that the sympathoenteric progenitor arises from the anterior region of the vagal crest (somites 1–5) and is dependent on Ret, while the sympathoadrenal progenitor arises from the posterior vagal crest (somites 6–7) and is not dependent on Ret (Durbec et al., 1996a).

Providing a more detailed explanation of the lineage of enteric neurons, Blaugrund et al. (1996) have suggested that there are at least two distinct enteric progenitor lineages. One displays sympathoadrenal markers (such as B2 antigen), is dependent on the MASH-1 transcription factor, and apparently gives rise to all serotonergic enteric neurons; another lineage does not express sympathoadrenal markers, is MASH-1 independent, and gives rise to nonserotonergic neurons of the gut. As PF cells express both B2 and MASH-1 (Clark et al., 1995a), they would appear to be developmentally related to serotonergic neurons of the gut. PF cells clearly appear to arise from a progenitor that has the potential to generate neurons. Further support for this prediction comes from the functional homology of NGF-induced endocrine–neuronal transdifferentiation seen for sympathoadrenal-derived chromaffin cells and PF cells (Unsicker et al., 1978; Doupe et al., 1985; Barasch et al., 1987a; Clark et al., 1995a). Finally, both PF cells and a subset of enteric neurons express serotonergic features (Fig. 1). These include production of 5-HT from tryptophan by tryptophan hydroxylase, neuronal 5-HT binding proteins, 5-HT_1 autoreceptors, and a neuronal 5-HT transporter (Nunez and Gershon, 1972; Barasch et al., 1987b; Clark et al., 1995a).

Neuronal Features of PF Cells

Thyroid PF cells have a neuroendocrine phenotype when in the thyroid gland. It is generally assumed that their major biological activity is to express the hormone calcitonin (CT) (Austin and Heath, 1981; Copp, 1992). As such, their physiological function is to sense increased serum calcium levels and secrete CT to reduce bone resorption and increase renal calcium secretion to lower serum calcium levels (McDermott and Kidd, 1987; Brown, 1991). While CT is dispensable in the normal adult, it is an effective therapeutic agent for some types of osteoporosis and bone disorders, e.g., Paget's disease (Austin and Heath, 1981; McDermott and Kidd, 1987; Copp, 1992). The gene that encodes CT also produces an alternative splicing product, calcitonin gene-related peptide (CGRP) (Rosenfeld et al., 1983). Whereas CGRP is mainly produced in neurons, PF cells do produce some CGRP that is released into the blood. The best characterized function of CGRP is to cause vasodilatation of peripheral and cerebral blood vessels (Brain et al., 1985; Marshall, 1992).

Although PF cells play a mostly endocrine role in vivo, they do have some intrinsic neuronal properties, such as 5-HT biosynthesis and expression of a neuronal 5-HT binding protein (Nunez and Gershon, 1972; Barasch et al., 1987b). Furthermore, PF cells have the potential to express even more neuronal features when placed in primary cultures or upon tumorigenesis. Primary cultures have been shown to extend neuritic processes in response to NGF (Barasch et al., 1987a; Clark et al.,

1995a) and plating on laminin (Nishiyama and Fujii, 1992) or a complex matrix (aneural chick hindgut) (Barasch et al., 1987a). One persistent problem with primary PF cell cultures has been poor cell attachment. Recently we have found that higher mol-wt poly-D-lysine (mol wt 70–150 kDa) greatly improved cell attachment, although we do not yet know whether the combination of this substratum with laminin is optimal for neurite extension. In the case of rat PF cell cultures, about 50% of the PF cells had processes greater than a cell body diameter (10–15 μm) after 5 d of NGF treatment (Clark et al., 1995a). Processes longer than 50 μm were seen on about 10% of the cells, with about 5% having processes over 100 μm (Fig. 2A).

NGF treatment has also been shown to increase expression of neurofilaments, CGRP, and 5-HT transporter mRNA and activity (Barasch et al., 1987a; Clark et al., 1995a). Most untreated thyroid PF cells did not express neurofilament immunoreactive material, nor is neurofilament immunoreactivity detected in thyroid PF cells in vivo. Scattered PF cells did show neurofilament immunoreactivity after several days in culture, indicating that some neurofilament expression occurs in culture. In contrast, NGF treatment for 5 d led to detection of NF-M neurofilament in about 85% of the PF cells (Clark et al., 1995a) (Fig. 2B). Immunolocalization of neurofilaments was confirmed by RT-PCR amplification of neurofilament RNA from the primary cultures (Fig. 2C).

Another indication of PF cell neuronal potential is the expression of MASH-1. As mentioned, MASH-1 is a marker of sympathoadrenal cells (Fig. 1). It is a transcription factor that has been shown to be present in neuronal precursors, but not in neurons after differentiation (Johnson et al., 1990; Lo et al., 1991; Guillemot and Joyner, 1993). Accordingly, mice lacking MASH-1 have autonomic ganglia with arrested neuronal differentiation (Guillemot et al., 1993). We have shown that MASH-1 is present in the rat PF cells. At present the only other example of MASH-1 expression in the postnatal animal is in olfactory neuroblasts (Guillemot and Joyner, 1993). Interestingly, once the cells are placed in culture and acquire more neuronal properties, MASH-1 is no longer detected (Clark et al., 1995a). Furthermore, as noted, MASH-1 is required for development of the serotonergic but not nonserotonergic neurons of the gut (Blaugrund et al., 1996). The presence of MASH-1 in neuroblasts and PF cells supports the interpretation that PF cells have retained neuronal potential. Likewise, the subsequent loss of MASH-1 in the primary cultures is consistent with neuronal differentiation in vitro.

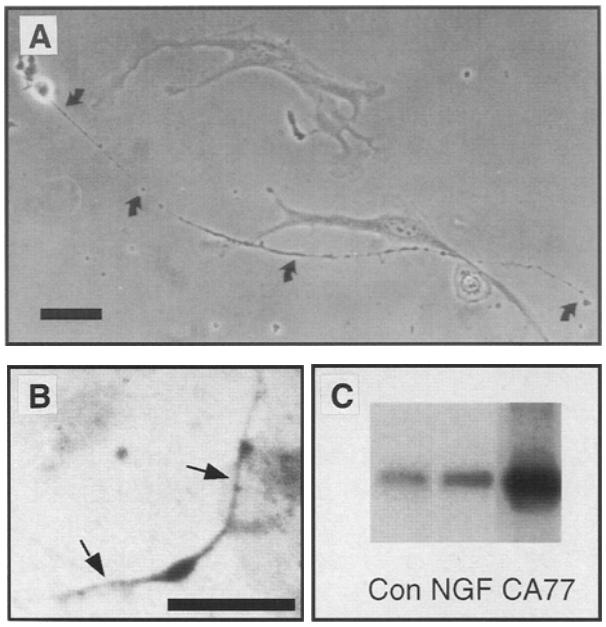


Fig. 2. Primary PF cells extend neurites and express neurofilaments. **(A)** Phase micrograph of PF cells cultured on fibronectin and laminin substratum and treated with NGF for 6 d. Neurite indicated by arrows extends approx 550 μm. Magnification bar is 50 μm. **(B)** Bright field micrograph of PF cell culture treated with NGF for 5 d, immunostained with a monoclonal antibody against 160 kDa neurofilament (NF-M), with detection by HRP-DAB staining. Neurites are indicated by arrows. Magnification bar is 50 μm. **(C)** Presence of NF-M RNA in PF cell cultures detected by RT-PCR. Total RNA was isolated from PF cell cultures treated for 5 d with NGF or vehicle alone (Con), reverse transcribed and PCR amplified using neurofilament NF-M specific primers. CA77 RNA was included as a positive control. A Southern blot of the RT-PCR products is shown.

Why do PF cells not become neuronal in vivo? Since crest cells are highly responsive to their microenvironments, it is reasonable that the answer lies in the thyroid environment. There is evidence that the thyroid both represses neuronal features and lacks the appropriate factors needed for neuronal differentiation. Supporting the active repression role, Jacobs-Cohen et al. (1994) have reported that triiodothyronine partially represses the development of neuronal morphology in PF cells. Therefore, it appears that PF cells may be induced to assume an endocrine phenotype by the thyroid environment, a situation analogous to the sympathoadrenal derived chromaffin cells in the adrenal environment.

The lack of an appropriate neurotrophic factor in the thyroid is indicated by the responsiveness of cultured PF cells to NGF. Yet NGF is not the determinant of the enteric neuronal phenotype (Baetge et al., 1990; Gershon et al., 1993; Schuchardt et al., 1994), so why do PF cells respond to NGF? One possibility is that NGF responsiveness may be a developmental holdover, still used by the trunk sympathoadrenal lineage (Anderson, 1993), but no longer physiological in the vagal sympathoadrenal branch. Alternatively, NGF may act on PF cells in vivo, based on the hypothyroidism seen in rats given NGF antiserum (Levi-Montalcini, 1982), as noted by Barasch et al. (1987a). Further support for a trophic role of NGF on PF cells is that NGF increased DNA synthesis and proliferation of cultured human PF cell tumors (Goretzki et al., 1987). Hence, although NGF is not the physiological neurotrophic factor for the vagal crest, it might play a role by contributing to PF cell survival.

It is now clear that glia-derived neurotrophic factor (GDNF) and the Ret tyrosine kinase receptor play key roles in vagal crest neuronal differentiation. Mutations that constitutively activate the Ret receptor have been genetically linked to multiple endocrine neoplasia, which includes PF cell tumors (Santoro et al., 1990; Hofstra et al., 1994; Mulligan et al., 1994; Santoro et al., 1995). As discussed below, PF cell tumors often have neuronal features. Likewise, loss of Ret function leads to Hirschsprung's disease (aganglionic megacolon), in which the enteric neurons do not develop (Edery et al., 1994; Romeo et al., 1994). Finally, the requirement of ret for terminal differentiation of enteric neurons has been demonstrated by studies on knock-out mice lacking the ret gene (Schuchardt et al., 1994). Genetic knock-out experiments have also elegantly shown the requirement for GDNF for enteric neurogenesis (Moore et al., 1996; Pichel et al., 1996; Sanchez et al., 1996). The connection between GDNF and Ret has now been solidified by the recent identification of Ret as the functional GDNF receptor (Durbec et al., 1996b; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996). Interestingly, GDNF does not directly bind Ret, but rather first must bind a lipid-anchored protein, GDNFR-α, which then activates Ret (Jing et al., 1996; Treanor et al., 1996). Hence, we predict that either the thyroid lacks GDNF or PF cells lack GDNFR-α, accounting for the lack of complete neuronal differentiation of PF cells in vivo. NGF treatment could induce a neuronal phenotype by activation of similar pathways to those used on GDNF activation of Ret. In this model, a balance of thyroid hormone actions and GDNF stimulation establishes the PF cell phenotype in the thyroid.

Neuronal Features of Cell Lines Derived from PF Cells

The presence of neuronal properties is common in PF cell tumors (medullary thyroid carcinoma [MTC]). Almost 70% of MTC tumors express neurofilaments (Wiedenmann et al., 1986) and 60% express neuron-specific enolase (Sikri et al., 1985). Likewise, MTC cells can acquire a neuritic morphology, as exemplified in the scanning electron micrograph of CA77 cells shown in Fig. 3. In addition, there is a shift from calcitonin to CGRP production in PF cell tumors (Rosenfeld et al., 1981). For example, while normal PF cells contain only 5% of CT/CGRP transcripts as CGRP mRNA (Amara et al., 1982), the CA77 PF cell line contains almost 90% of the transcripts as CGRP mRNA (Russo

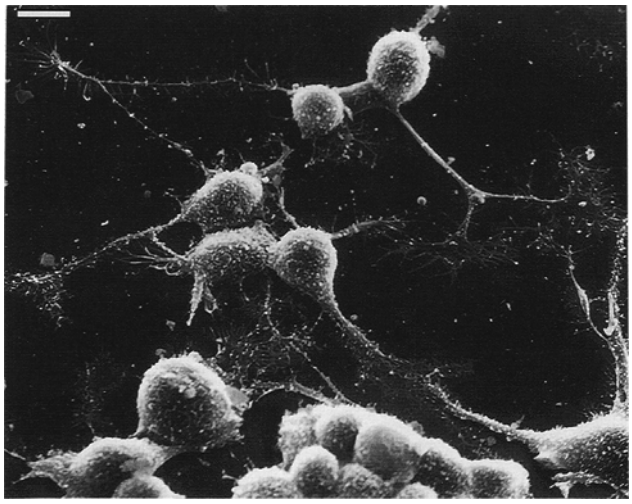


Fig. 3. Electron micrograph of neurites in CA77 PF cells. Scanning electron micrograph of CA77 cells. Micrograph kindly provided by Dr. Lois Tverberg. Note extensive neurite formation with abundant filopodia. Magnification bar at upper left is 10 μm.

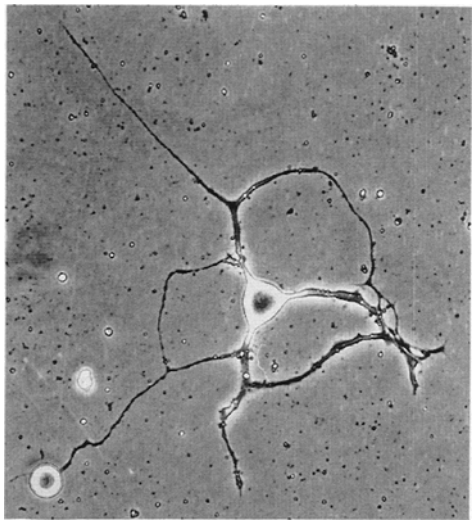


Fig. 4. Neuronal properties of CA77 PF cells. Phase contrast micrograph illustrating the extension of neurites from a single CA77 cell. The morphological, biochemical, and serotonergic properties of CA77 cells that are consistent with a neuronal-like phenotype are as follows: Morphology: neurites and growth cones, clear and dense core vesicles; biochemical: neurofilaments (NF-L, NF-M, NF-H), NF-associated protein (3A10), synaptophysin, neuronal glycolipid (LA4), High CGRP/CT ratio, N-type calcium channels, TTX-sensitive sodium channels, and MASH-1, Emx-1, and Brn-3 transcription factors; serotonergic: tryptophan hydroxylase mRNA, regulated 5-HT secretion, 5-HT1B autoreceptor, 5-HT transporter mRNA, SSRI-sensitive 5-HT reuptake.

et al., 1988). This qualitative bias toward CGRP mRNA production is also seen in NGF treatment of PF cells in primary culture (Barasch et al., 1987a). However, the PF cell lines differ in the degree of their neuronal phenotypes (Clark et al., 1995b; Russo and Lanigan, 1996). Among the MTC cell lines, the CA77 cells are the most neuronal in culture.

The CA77 cell line was established by Bernard Roos and colleagues in 1980 from a serially passaged rat MTC (Muszynski et al., 1983). The CA77 cells express both morphological and biochemical traits characteristic of neurons (Fig. 4) (Russo et al., 1992; Russo and Lanigan, in press). They have neurites well over 100 μm long and express all three neurofilaments: NF-L, NF-M, and NF-H. The neurites and cell bodies contain both clear and dense core vesicles. The cells also express other markers seen in neural cells, including a neurofilament-associated protein (synaptophysin), a neuronal glycolipid, and the transcription factors MASH-1, Emx-1, and Brn-3 (Russo et al., 1992; unpublished data). Another measure of the neural state is the high ratio of CGRP relative to calcitonin mRNA. Finally, electrophysiological recordings of the CA77 cells have revealed neural-type ion channels. There is an ω-conotoxin sensitive N-type Ca^{2+} current and a tetrodotoxin-sensitive Na^{+} current. In addition, there are a number of serotonergic properties as discussed below.

The TT PF cell line was established by Steve Baylin and colleagues from a human MTC (Leong et al., 1981). In contrast to the neuritic morphology of CA77 cells, the TT cell line has a more fusiform morphology under normal growth conditions. However, the TT cells express many serotonergic properties, and neuronal differentiation can be enhanced by a reduced serum medium (Tamir et al., 1989). Under these conditions, there was a more neuritic morphology, increased neurofilament,

monoamine oxidase-A (MAO-A), and serotonin-binding protein (SBP) immunoreactivity. Hence both CA77 and TT PF cell lines provide useful model systems.

Why do PF cell tumors and cell lines express neuronal-like properties? We propose that activation of the Ret receptor upon tumorigenesis induces these neuronal properties (Fig. 1). The connection between Ret activation and neuronal differentiation of PF cells is further supported by observations that inhibiting the growth of the CA77 and TT PF cells by different means causes at least partial repression of the neuronal properties (Cote and Gagel, 1986; Nakagawa et al., 1987; Nelkin et al., 1989; Russo et al., 1992). For example, dexamethasone-treatment inhibits the growth rate of CA77 cells 10-fold with partial retraction of neurites, decreased neurofilament mRNA levels, and increased production of CT relative to CGRP mRNA (Russo et al., 1992; Clark et al., 1995b).

Serotonergic Properties of PF Cells and Cell Lines

To function as models for serotonergic neurons, PF cells should possess the fundamental features of serotonergic neurons. They should be capable of synthesizing and releasing 5-HT in a regulated manner, have a feedback mechanism for sensing extracellular 5-HT (an autoreceptor), and be able to remove the extracellular 5-HT via a transporter. These criteria have now been demonstrated for both PF cell lines and primary PF cell cultures (Nunez and Gershon, 1972; Barasch et al., 1987b; Tamir et al., 1989; Tamir et al., 1990; Clark et al., 1995a).

The pioneering work in this area was performed by Nunez and Gershon (1972) almost 25 yr ago using PF cell cultures from bats to document the synthesis of 5-HT from tryptophan. Subsequent studies by Gershon, Tamir, and colleagues with sheep PF cells demonstrated that 5-HT was stored in granules bound to a neuronal 5-HT binding protein (Barasch et al., 1987b). More recently, serotonergic properties have also been shown using primary rat PF cell cultures and the rat CA77 and human TT cell lines. The TT MTC cell line has been extensively characterized by Tamir and coworkers and shown to synthesize and store 5-HT and release it in response to extracellular calcium or thyroid stimulating hormone (Tamir et al., 1989; 1990). In addition, TT cells contain a fluoxetine-inhibitable reuptake mechanism, MAO-A, 45 kDa, and 56 kDa SBPs that are apparently identical to those in the CNS, and 5-HT storage vesicles with a reserpine sensitive biogenic amine transporter (Tamir et al., 1989). Interestingly, 5-HT release from PF cells forms a paracrine regulatory loop that can control thyroid hormone release from the adjacent follicular cells (Tamir et al., 1992). Many of these properties are associated with serotonergic neurons, but not with non-neuroectodermal cells that store 5-HT, such as platelets and mast cells.

The CA77 cells, as well as primary rat PF cells, have recently been shown to possess serotonergic properties (Fig. 5) (Clark et al., 1995a; 1995b). The CA77 and primary PF cells demonstrated 5-HT immunostaining. 5-HT storage and release from the CA77 cells was documented by HPLC. Both CA77 and primary PF cells also expressed tryptophan hydroxylase mRNA. CA77 cells contain 1.8 and 4.0 kb TPH mRNAs (Clark et al., 1995b), which are the same sizes as identified in the brain by Darmon et al. (1988). Furthermore, from both primary PF cells and CA77 cells, we have detected the presence of mRNA encoding the rat 5-HT_{1B} autoreceptor (Fig. 5B). This receptor is generally found on axons (Boess and Martin, 1994). As discussed below, pharmacological evidence has confirmed the expression of a functional 5-HT_{1B} autoreceptor on the CA77 cells. Finally, 5-HT transporter mRNA and activity was found in the CA77 cells and primary cultures. The transporter sequence was identical to that found in the brain (Blakely et al., 1991). Interestingly, NGF treatment of the primary cultures was needed to induce transporter activity and, apparently, transporter mRNA. 5-HT transporter activity was measured by sensitivity to sertraline, a selective 5-HT reuptake inhibitor. Sertraline reduced uptake of ^{3}H-5-HT into CA77 cells to about 7% of uptake in the absence of the inhibitor (Fig.

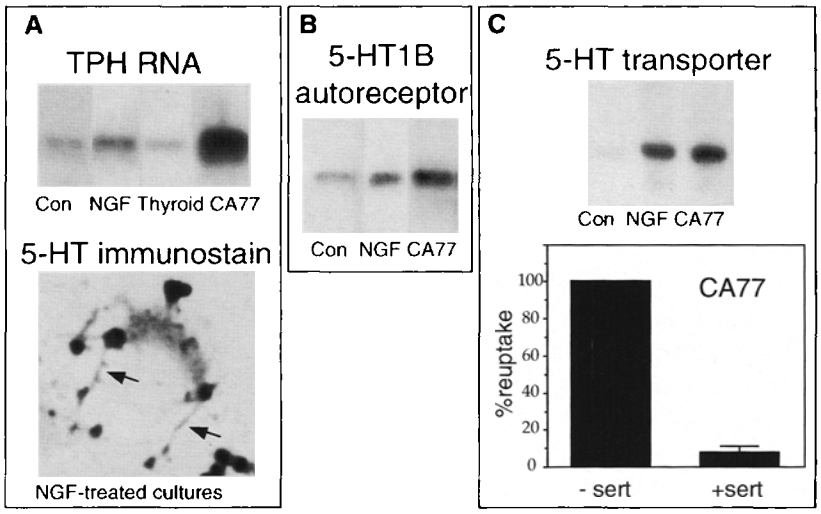


Fig. 5. Serotonergic properties of PF cells and CA77 cells. **(A)** PF cells produce tryptophan hydroxylase (TPH) mRNA and contain 5-HT. Total RNA was isolated from either primary PF cell cultures treated with NGF for 5 d (NGF), vehicle alone (Con), directly from rat thyroid gland (Thyroid), or CA77 cells (CA77). RNA was reverse transcribed and PCR amplified using TPH specific primers. A Southern blot of the RT-PCR products is shown **(top)**. Bright field micrograph of HAP-DAB stained PF cell culture treated with NGF for 5 d, immunostained with antiserum against 5-HT **(bottom)**. Neurites are indicted by arrows. **(B)** Total RNA was prepared as in (A), reverse transcribed, and PCR amplified using 5-HT_{1B} specific primers. A Southern blot of the RT-PCR products is shown. **(C)** Total RNA was prepared as in (A), reverse transcribed, and PCR amplified using 5-HT transporter specific primers **(top)**. A Southern blot of the RT-PCR products is shown. Uptake of ^{3}H-5-HT at 37°C was determined in the presence and absence of sertraline for CA77 cells **(bottom)**. Sertraline reduced 5-HT uptake to 7.2 ± 4.1% of control values.

5C). When the PF cell cultures were tested, sertraline repressed 5-HT uptake about two-fold in NGF-treated PF cells, and had only a negligible effect on control cultures (Clark et al., 1995a). Since the PF cell cultures were not pure, the sertraline-resistant uptake in the NGF-treated cells was most likely a result of nonspecific transport by contaminating fibroblasts. Hence, normal PF cells have the potential to express the transporter that is constitutively expressed in the PF cell lines.

Applications of Serotonergic Properties of PF Cells

Transcriptional Regulation of 5-HT Biosynthesis

Serotonergic dysfunction is believed to underlie a number of psychiatric disorders, particularly major depressive disorder and obsessive-compulsive disorder (Blundell, 1984; Azmitia and Whitaker-Azmitia, 1991; Jacobs and Azmitia, 1992). Therapeutic approaches have concentrated on modulating 5-HT levels, primarily through the use of selective 5-HT reuptake inhibitors, of which fluoxetine (Prozac) is the most widely used (Owens and Nemeroff, 1994). Since PF cells in culture express the TPH gene required for 5-HT biosynthesis, the 5-HT_{1B} autoreceptor, and 5-HT transporter, they offer an opportunity to study molecular interactions between the blockade of transporter function with uptake inhibitors and TPH biosynthetic and autoreceptor activities. Likewise, the effect of other agents believed to affect 5-HT levels can potentially be determined.

Likely regulators of TPH biosynthetic activity are glucocorticoid (GC) hormones. Glucocorticoids have a multitude of activities,

primarily as mediators of the stress response, and are widely recognized to alter mood in humans. Clinical use of high dosages of GCs for prolonged duration may induce features of depression, including dysphoria, anxiety, irritability, and occasional psychotic episodes (McEwen, 1987; Satel, 1990). Similar symptoms may be observed in patients with Cushing's syndrome (McEwen, 1987). Likewise, a significant number of depressed individuals possess chronically elevated cortisol levels (Caroll, 1982). However, despite the clear clinical connection between glucocorticoids and behaviors associated with 5-HT, there have been contradictory reports on the effects of chronic GC treatment on 5-HT levels (Chaouloff, 1993). To resolve this issue, we chose to measure TPH mRNA levels, since TPH is the rate-limiting enzyme in 5-HT biosynthesis, and gene transcription is a reasonable target for long-term regulation of 5-HT biosynthetic potential. Furthermore, the mRNA levels would not be as susceptible to the confounding influences that could acutely modulate 5-HT levels during experimental manipulations.

We have compared the effect of the synthetic glucocorticoid dexamethasone on TPH mRNA levels in the raphe nuclei of the brain and the thyroid gland in vivo with the effect on CA77 PF cells in vitro. While TPH mRNA can be detected by northern blots of CA77 cell RNA (Clark et al., 1995b), it is an exceedingly rare transcript in the raphe nuclei of the brainstem, and is extremely difficult to detect by northern or ribonuclease protection analyses (Darmon et al., 1986; Dumas et al., 1989). To overcome this difficulty, we established a competitive RT-PCR assay. Competitive RT-PCR is an accepted quantitative assay, which uses a competitor template RNA that shares the same primer sites as the target RNA (in this case TPH), yet can be distinguished from the target amplification product (Gilliland et al., 1990; Siebert and Larrick, 1992). When the target and competitor amplification products are equal, then the concentration of target can be calculated since the amount of competitor is known. Chronic treatment of rats for 7 d with dexamethasone decreased TPH mRNA levels sixfold in the raphe nuclei (Clark and Russo, submitted for publication).

However, the study of TPH mRNA levels in viva reveals little about mechanistic details. The decrease in mRNA levels may be a result of either transcriptional or posttranscriptional mechanisms and the effect may be a secondary consequence of dexamethasone action on other cells. Testing the effect of dexamethasone treatment on the CA77 cells then could help to resolve these issues. Furthermore, CA77 cells provided a useful validation of the competitive RT-PCR assay since the results could be confirmed by hybridization blots. CA77 cells were treated with dexamethasone or vehicle alone for 1–5 d. At 10 n*M* or greater concentrations, TPH mRNA levels were reduced five- to sixfold, as determined by RNA slot blots (Clark et al., 1994). When the same RNA samples were measured by competitive RT-PCR, TPH mRNA levels were reduced by fivefold (Fig. 6A,B). Since dexamethasone represses TPH mRNA levels in a homogeneous cell line, this indicates that GCs are capable of directly regulating TPH mRNA levels. As further evidence that the CA77 cells accurately reflect the in vivo conditions, we measured a similar repression of TPH mRNA levels in the thyroid gland (Fig. 6C).

The functional significance of the GC-mediated repression of TPH mRNA levels is that it would decrease the potential 5-HT biosynthetic capacity. This down-regulation suggests the existence of a classic homeostatic feedback loop. Previous studies have shown that short-term treatment with GCs, as well as a variety of acute stresses, can increase TPH activity in rats (Gal et al., 1968; Azmitia and McEwen 1974; Archer, 1982; Azmitia et al., 1993). Since increased 5-HT in the CNS is known to induce cortisol release (Chaouloff, 1993), this would create a positive feedback loop. The down-regulation of TPH mRNA levels is consistent then with an eventual termination of this stimulatory loop under chronic stress conditions. The similar down-regulation of TPH in the raphe, thyroid, and CA77 cells demonstrates the utility of PF cells for further studies into the mechanisms underlying TPH gene expression.

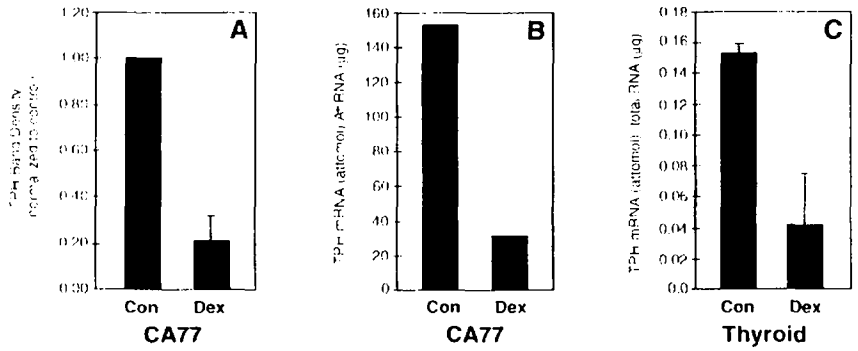


Fig. 6. Dexamethasone repression of TPH mRNA levels in CA77 cells and the thyroid. **(A)** CA77 cells were treated with either 100 n*M* dexamethasone (Dex) or vehicle alone (Con) for 5 d. As measured by RNA slot blot analysis, dexamethasone decreased TPH mRNA levels in CA77 cells by five- to sixfold. Equivalent results are obtained following 1 d of treatment. **(B)** A sample of the same CA77 RNA was examined by competitive RT-PCR, which also revealed a five- to sixfold decrease in TPH mRNA levels. **(C)** Thyroid TPH mRNA concentrations were determined by competitive RT-PCR following 7 d of treatment with either 2.5 mg/200 g body weight dexamethasone sodium phosphate (Dex) or vehicle alone (Con). TPH mRNA levels in thyroid tissue were decreased by about fourfold.

Regulation of Calcitonin/CGRP Gene Transcription

A particularly useful application of a cell line is the analysis of *cis*- and *trans*-acting transcription factors, owing to the cell type homogeneity and ability to measure promoter activity by transient transfection assays with reporter genes. The PF cells are an especially appropriate system for these types of studies on neuropeptides, since PF cells and cell lines express a large number of neuropeptide genes, including CT, CGRP, somatostatin, NPY, CCK, and substance P (unpublished data) (VanNoorden et al., 1977; Rosenfeld et al., 1983; Deschenes et al., 1984; Cremins et al., 1992), similar to the host of peptides expressed in enteric neurons (Baetge et al., 1990; Lindh and Hokfelt, 1990).

As mentioned, the hallmark of PF cells is the expression of the CT/CGRP gene, which is also expressed in a subset of neurons, including enteric neurons. The CT/CGRP gene is regulated by a variety of agents including cAMP and phorbol esters (deBustros et al., 1986; Monia et al., 1995; Supowit et al., 1995), vitamin D (Naveh-Many and Silver, 1988; Peleg et al., 1993), nerve growth factor (Lindsay and Harmar, 1989; Watson and Latchman, 1995), retinoic acid (Lanigan et al., 1993), and glucocorticoids (Muszynski et al., 1983; Russo et al., 1988; Tverberg and Russo, 1992; Supowit et al., 1995). Regulation by cAMP appears to be especially complex, involving multiple cAMP response elements (CREs) that are modulated in a cell-specific manner (deBustros et al., 1990; deBustros et al., 1992; Monia et al., 1995). In general, CREs bind *trans*-acting factors that are activated by a phosphorylation cascade initiated by protein kinase A (PKA). This phosphorylation cascade has pleiotropic effects on cells, for example leading to calcium channel phosphorylation. In addition to regulating CT/CGRP transcription, cAMP also increases secretion of both CT and CGRP from thyroid PF cell lines (unpublished data) (deBustros et al., 1986). Since 5-HT autoreceptors can modulate PKA activity, and possibly other signal transduction pathways (Boess and Martin, 1994), it seems reasonable to predict that 5-HT might also regulate CT/CGRP gene expression, as discussed below.

Studies in transgenic mice have demonstrated that the 5' flanking DNA of the rat CT/CGRP gene contains a cell-specific enhancer that targets expression to thyroid PF cells, and possibly neurons (Stolarsky-Fredman et al., 1990;

Baetscher et al., 1991). Further mapping studies have used transient transfection assays with the PF cell lines. Mapping of the human (Peleg et al., 1990; Ball et al., 1992) and rat (Stolarsky-Fredman et al., 1990; Tverberg and Russo, 1992; Tverberg and Russo, 1993) gene has identified helix-loop-helix (HLH) binding sites required for cell-specific expression. In the rat gene, we have demonstrated that a single HLH site is responsible for most, if not all, of the cell-specific enhancer activity (Tverberg and Russo, 1993). Interestingly, this site cannot function on its own, but requires an adjacent overlapping octamer-binding motif. The functional significance of both the HLH and octamer motifs has been demonstrated by a series of point mutations; the proteins that bind these sites have been characterized by electrophoretic mobility shift assays (Tverberg and Russo, 1993). Two cell-specific bands were identified: HB1, corresponding to an HLH protein; and OB2, corresponding to an octamer-binding factor. In addition, binding of the ubiquitous Oct-1 protein was documented by antibody supershift assays. A candidate for the cell-specific HB1 complex was the MASH-1 protein, which, as discussed, is expressed in rat PF cells as well as in the human TT PF cell line (Ball et al., 1993). Cotransfection of a MASH-1 expression vector yielded a small but significant increase in the enhancer activity (Tverberg and Russo, 1993). Despite this circumstantial evidence, it remains to be proven that MASH-1 is the in vivo regulator. Experiments currently underway using MASH-1 knockout mice should clarify this issue.

A fundamental feature of the CT/CGRP enhancer is that it involves synergism between the HLH and octamer-binding motifs for full activity (Fig. 7) (Tverberg and Russo, 1993). The presence of only one motif yields less than two- to fourfold activation, in contrast to the 20–30-fold activation seen with both motifs. Likewise, when either site was mutated or separated by insertion of 1, 5, or 10 bp, the activity was greatly reduced (unpublished data) (Tverberg and Russo, 1993). While this is the first report of synergism between HLH and

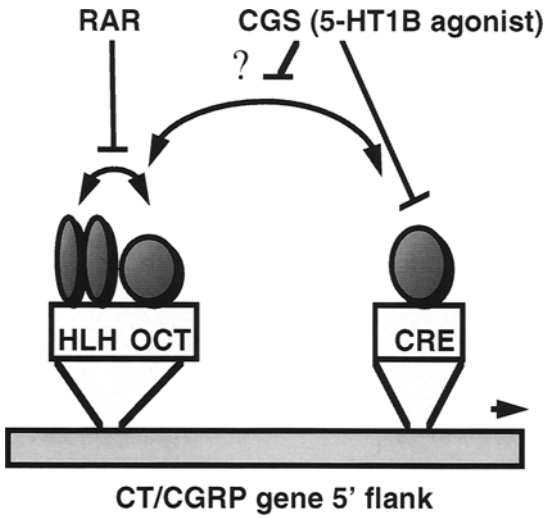


Fig. 7. Activation and repression of the CT/CGRP enhancer. The 5' flanking sequence of the rat CT/CGRP gene is shown with the 18-bp cell specific enhancer sequence and cAMP responsive element (CRE). The cell specific enhancer contains sites for helix-loop-helix (HLH) and octamer (Oct) binding proteins. Activation of the enhancer involves synergism between the HLH and octamer-binding factors. The enhancer is repressed by protein–protein interactions with the retinoic acid receptor (RAR). Preliminary data indicate that the $5HT_{1B}$ receptor agonist, CGS, can repress both the CRE and the enhancer activity, although the mechanism remains to be established.

octamer-binding proteins, it is likely to be followed by many other examples. In general, synergism between transcription factors is an increasingly common theme in the control of gene expression (Struhl, 1991). This may be especially true for cell-specific gene expression. For example, synergy between an HLH and LIM-homeodomain protein has been shown to be important for cell-specific control of the insulin gene (German et al., 1992); HLH-AP1 interactions have been reported to be important for the tyrosine hydroxylase gene (Yoon- and Chikaraishi, 1992).

These synergistic activities are ideal targets for modulating gene transcription by external signals. In support of this prediction, we have shown that the CT/CGRP cell-specific enhancer is repressed by a glucocorticoid agonist (Tver-

berg and Russo, 1992) and retinoic acid (Lanigan et al., 1993). Nuclear receptors repress transcription by several mechanisms (Beato et al., 1995). We have evidence that retinoic acid repression is not mediated by a direct DNA binding mechanism, but rather results from inhibitory protein–protein interactions (Lanigan et al., 1993). In the case of vitamin D repression, Peleg et al. (1993) have shown that repression requires both the CRE and the cell-specific enhancer. Recent cloning of a nuclear receptor interacting protein (CBP) that also interacts with CRE binding protein (Kamei et al., 1996) supports the intriguing possibility that there might be bridging proteins between the cell-specific enhancer and the CRE.

Serotonergic Regulation of CT/CGRP Gene Expression

CGRP has been well characterized as a potent dilator of peripheral and cerebral blood vessels (Brain et al., 1985; McCulloch et al., 1986; Preibisz, 1993). In particular, a crucial role of CGRP has been revealed in the control of cerebral vascular tone. High levels of CGRP are also associated with vascular headaches (Edvinsson and Goadsby, 1994). Vascular headaches include migraines and cluster headaches. The involvement of CGRP with headaches is of particular significance owing to the prevalence of this disorder. About 50 million Americans visit a doctor every year because of headaches, and headache is one of the most common complaints reported to primary care physicians (Diamond, 1994). The vascular group of headaches affect 16% of the population and account for 50% of the headaches referred to specialists (Diamond, 1994). Hence, there is a need for improved therapeutic and preventative measures for vascular headaches. One approach toward this goal is to develop better means for modulating CGRP levels.

Plasma levels of CGRP are increased in all three major vascular headache categories: migraine with aura, migraine without aura, and cluster headache (Edvinsson and Goadsby, 1994). Functional studies have demonstrated that administration of exogenous CGRP causes vasodilatation of cerebral arteries in vitro and *in situ* and that it is the most potent vasodilator among the perivascular peptides (McCulloch et al., 1986). The CGRP fibers that innervate the cerebral blood vessels have been traced to the trigeminal ganglia (McCulloch et al., 1986). In addition to causing vasodilatation, trigeminal nerves provide the only sensory (pain-sensitive) innervation of the cranial vasculature (Edvinsson and Goadsby, 1994). However, a fundamental drawback of studying the regulation of CGRP in the trigeminal is its heterogeneity. CGRP immunofluorescence was detected in <1% of trigeminal cells *in situ* (Rosenfeld et al., 1983). This heterogeneity highlights the advantage of the CA77 PF cell line for molecular studies.

Recently, a 5-HT type-1 (5-HT_1) receptor agonist, sumatriptan, was reported to be an effective therapeutic agent for all three types of vascular headaches (Edvinsson and Goadsby, 1994). Following treatment with sumatriptan, elevated serum CGRP levels were returned to normal, coincident with relief of the symptoms (Goadsby and Edvinsson, 1991; Goadsby and Edvinsson, 1993). Likewise, nonheadache associated release of CGRP upon stimulation of trigeminal nerves is also attenuated by sumatriptan (Goadsby et al., 1988; Buzzi et al., 1991). Based on pharmacological evidence, sumatriptan is believed to act through the human 5-HT_{1D} receptor (Schoeffter and Hoyer, 1989), which is widely expressed throughout the body, including in the trigeminal ganglia (Rebeck et al., 1994). The rat 5-HT_{1B} receptor is the functional homolog of the human 5-HT_{1D} receptor, and it is also present on trigeminal ganglia (Briunvels et al., 1992). Both of these receptors act by decreasing intracellular cAMP levels (Boess and Martin, 1994; Peroutka, 1995), which, as mentioned, could potentially inhibit both CT/CGRP transcription and secretion. This may account for the relatively long-term suppression of migraine and CGRP levels by sumatriptan beyond an initial inhibition of secretion.

While we have only begun using the PF cells to measure sumatriptan actions on the

CT/CGRP gene, our preliminary results are encouraging. We have found that treatment of the cells with CGS-12066A maleate (CGS) leads to a down-regulation of CGRP mRNA levels and promoter activity (Durham and Russo, manuscript in preparation). CGS is a novel pyrroloquinoxaline with selectivity for 5-HT_{1B} receptors and is more resistant to breakdown by MAO than sumatriptan (Neale et al., 1987). CGS treatment led to an apparent decrease in cAMP-stimulation of the CT/CGRP gene. Most intriguingly, long-term treatment with CGS (12–16 h) appears to repress promoter activity through the cell-specific enhancer. The apparent link between the cell-specific enhancer and CRE is in agreement with observations that the cAMP responsiveness has cell-specificity (deBustros et al., 1990; Monia et al., 1995) and that vitamin D repression requires both the upstream enhancer and CRE (Peleg et al., 1993). These preliminary observations on CGS-12066 actions demonstrate the utility of PF cells for studying the mechanisms of serotonergic antimigraine drugs.

Summary

Thyroid PP cells share a common ontogeny with enteric serotonergic neurons, and can be induced to express a neuronal phenotype following NGF treatment. PF cell lines share these serotonergic neuronal features. We have proposed that these properties are the result of activated Ret kinase, analogous to the actions of Ret in enteric neurogenesis. Finally, the PF cell lines provide an amenable system to examine transcriptional regulation of 5-HT biosynthesis and serotonergic regulation of the CGRP gene. Further applications using thyroid PF cell lines should advance our understanding of the molecular control mechanisms and actions of serotonergic neurons.

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