Neurotrophic factor GDNF and cAMP suppress glucocorticoid-inducible PNMT expression in a mouse pheochromocytoma model.

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Chromaffin cells and their precursors exhibit considerable plasticity of phenotype. In the developing adrenal medulla, specific arrays and timing of environmental cues contribute to the commitment of sympathoadrenal precursors to the chromaffin cell lineage. Those cues include neurotrophic factors, glucocorticoids, and intracellular messengers, including cAMP. The selective induction of developmental programs in pluripotent cells likewise implies that competing or opposing programs must necessarily be repressed or silenced at the genomic level. We hypothesize that neuronal inductive cues exert opposite, repressive influences on the glucocorticoid-stimulated profile of endocrine marker expression in those sympathoadrenal cells capable of becoming adrenergic chromaffin cells. We further report here that mouse pheochromocytoma cells (MPCs) exhibit cooperative silencing of phenylethanolamine N-methyltransferase (PNMT) expression in cell culture. This effect may serve as a model for developmentally regulated expression of PNMT in the manner of differentiating sympathoadrenal precursors *in vivo*.

Mouse pheochromocytoma (MPC) lines MPC 862L and 10/9 CRC1 (10/9s) were initially characterized by expression of PNMT and stimulus-induced secretion of epinephrine[1]. Additionally, neurotrophic factors and glucocorticoids (GCs) were found individually to elicit specific morphological responses. In MPC 862 cells, the neurite production evoked by treatment with glial cell-line-derived neurotrophic factor GDNF and cAMP distinctly contrasts with the characteristic polygonal or rounded endocrine phenotype of control (Figure 1, top).

Because these morphological changes are likely consequences of developmental commitment decisions, the objectives of this study were to resolve 1) whether expression of PNMT is regulated analogously in MPC cells and adrenal chromaffin cells and 2) whether initiation of a neuronal phenotype in MPC cells prohibits subsequent induction of an endocrine phenotype. GDNF and cAMP influences on the dexamethasone (DEX)-responsiveness of the PNMT gene were

evaluated by measuring steady state levels of PNMT mRNA and transcription from the PNMT promoter in MPC cell lines. Treatments were performed on consecutive days (Fig. 1, bottom) with cultures harvested 24 hr after beginning the third treatment interval.

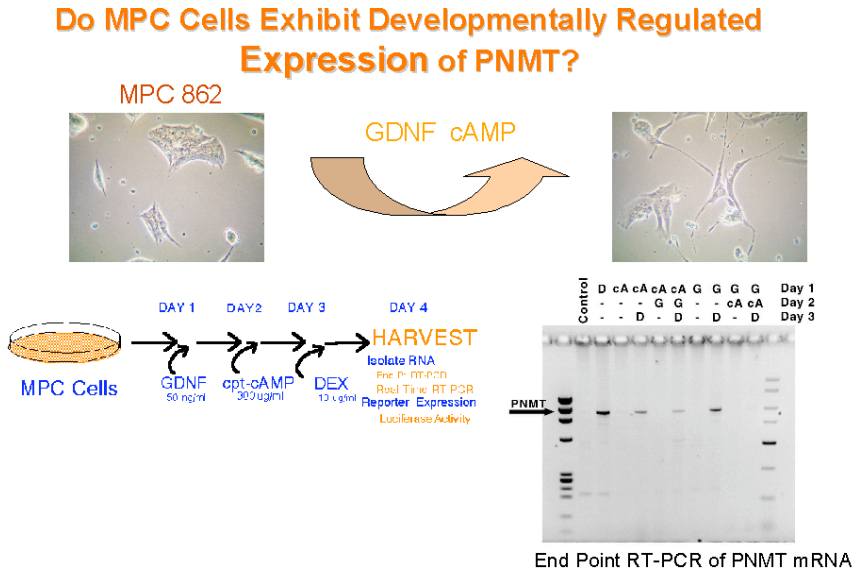

Figure 1. Neurotrophic factor GDNF (50 ng/ml) and cpt-cAMP (300 g/ml) elicit morphological changes including neurite extension in MPC 862 L cells (top). These neuronal inductive agents also significantly influence expression of PNMT mRNA in MPC cells. When MPC cells are treated sequentially with GDNF, cAMP, and DEX (bottom left), then harvested for isolation of total RNA, end point RT-PCR (bottom right) reveals that the induction of PNMT mRNA by DEX (D) is not observed following treatment with cAMP (cA) or GDNF (G). Notably, combined treatment with cAMP and GDNF prior to addition of DEX completely prevents induction of detectable levels of PNMT mRNA.

Semi-quantitative RT-PCR approaches quantified PNMT mRNA as the primary index of adrenergic response following

treatments with GDNF, cAMP, and DEX. End point RT-PCR revealed that DEX induced marked increases in PNMT mRNA levels in MPC 862s and in MPC 10/9s (Figure 1). No induction (<1-fold relative to control) occurred with GDNF or cpt-cAMP alone. However, when GDNF and cAMP together were evaluated for ability to alter responsiveness to DEX, treatment first with GDNF then with cAMP completely suppressed steady state levels of PNMT mRNA. (Individually, these neuronal agents reduced DEX-induction of PNMT mRNA by ~90% each). These neuronal cues, alone and in combination, therefore, effectively abrogated glucocorticoid-mediated induction of the endocrine phenotype in MPC cells.

To investigate the mechanism by which GDNF and cAMP alter DEX responsiveness of PNMT expression, stable transfectants of MPC 10/9 cells expressing ~ 1kb of the PNMT promoter were treated as before (Fig.1). While DEX alone elevated luciferase reporter gene activity approximately three-fold (indicating that GC signaling in MPCs analogous to previously studied chromaffin cells[2,3]), prior treatment with GDNF and cAMP significantly altered the magnitude of subsequent DEX induction. Sequential treatment of MPC 10/9s with GDNF, cAMP, then DEX produced ~50% reduction relative to DEX alone. Thus, neurotrophic factors and cAMP modulate PNMT transcription in MPC cells by decreasing the magnitude of GC induction.

Additional analyses of the 5' flanking region by transient transfection of nested deletion constructs revealed that the greatest effects of GDNF and cAMP on DEX inducibility of PNMT expression were achieved using the 'full length' ~1 kb promoter. Truncation of distal promoter regions successively reduced the magnitude of suppression up to the region of the GRE at -522 (For constructs in which DEX induction cannot occur, e.g. 3' to the GRE and with a mutated GRE, tests for DEX suppression did not differ from control expression.) These analyses demonstrate that the PNMT GRE plays a necessary, but not sufficient role in the transcriptional

aspects of this control mechanism. The greater responses observed with longer constructs, furthermore, implicate the participation of sequences in the distal 5' promoter of this gene.

In summary, MPC cells may provide important insight into regulatory mechanisms governing sympathoadrenal differentiation of neuronal and endocrine cells. In a manner that may parallel developmental responses for chromaffin cells *in vivo*, treatment of MPC cells with neurotrophic factor and cAMP effectively suppresses the induction of a specific gene, PNMT, that is critical to the acquisition of an endocrine phenotype. Moreover, the distinction between 100-fold induction of steady state mRNA levels vs. 3-fold stimulation of promoter activity argues for a regulatory mechanism in addition to transcriptional control. We have previously demonstrated that the cAMP-inducing peptide PACAP selectively enhances PNMT mRNA degradation[4] (while not influencing TH or DBH messenger half lives). That finding, coupled with our present observation that GDNF plus cAMP effectively prevent PNMT mRNA accumulation in MPC 862 and 10/9 cells, suggests that PNMT mRNA stability may represent an important locus for developmental regulation of the adrenergic phenotype.

The results of this study argue that the neuronal program of development predominates over the endocrine program of sympathoadrenal differentiation in MPC lines in a manner analogous to that observed in adrenal chromaffin cells. Furthermore, because these experiments have delineated both the individual and combined influences of specific neuronal and endocrine cues, it may additionally be noted that MPC cell lines 862 and 10/9 provide appropriate model systems for investigating the roles of intracellular signals in sympathoadrenal commitment.

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