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Autoregulation of Neurogenesis by GDF11

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Summary

In the olfactory epithelium (OE), generation of new neurons by neuronal progenitors is inhibited by a signal from neurons themselves. Here we provide evidence that this feedback inhibitory signal is growth and differentiation factor 11 (GDF11). Both GDF11 and its receptors are expressed by OE neurons and progenitors, and GDF11 inhibits OE neurogenesis in vitro by inducing p27Kip1 and reversible cell cycle arrest in progenitors. Mice lacking functional GDF11 have more progenitors and neurons in the OE, whereas mice lacking follistatin, a GDF11 antagonist, show dramatically decreased neurogenesis. This negative autoregulatory action of GDF11 is strikingly like that of its homolog, GDF8/myostatin, in skeletal muscle, suggesting that similar strategies establish and maintain proper cell number during neural and muscular development.

Introduction

The sizes of neuronal populations are critical determinants of nervous system function and are under tight genetic control (Williams, 2000). In the vertebrate nervous system, the gradual slowing and then cessation of progenitor cell proliferation toward the end of embryonic development (Caviness et al., 1995; Kauffman, 1968) suggests that neurogenesis is under some form of negative control. Experiments on model systems support the idea that differentiated neurons produce signals that feed back to inhibit the generation of new neurons by neuronal progenitors (Mumm et al., 1996), but the molecules that mediate such effects in vivo have not been identified. Elucidating such negative growth signals is likely to be very important, not only for understanding nervous system development, but also for devising strategies to deal with brain injury and aging, in which persistent growth-inhibitory signals could thwart attempts to promote regeneration.

To understand the molecular regulation of neurogenesis, we study a model neuroepithelial tissue, the olfactory epithelium (OE) of the mouse. The OE is morphologically and functionally similar to the neuroepithelia that generate the nervous system, but has significant advantages as a system for study. The OE is simpler, producing only one major type of neuron, the olfactory receptor neuron (ORN). Significantly, the OE retains both its epithelial morphology and the ability to generate neurons throughout life (Calof et al., 1996a). In addition, application of a variety of experimental approaches has allowed identification of different cell stages in the ORN lineage and revealed important features of the regulation of neurogenesis in this system. Among these is the finding that neuron production in the OE is governed by negative signals, which play at least as important a role as positive signals in this system (Calof et al., 2002).

In the OE, tight regulation of neurogenesis serves to maintain the size of its neuronal population at a particular level. Thus, in normal animals in which ORNs are constantly dying in low numbers (due to disease or injury), a low level of neurogenesis is constantly replacing them. If experimental manipulations are used to induce death of large numbers of ORNs, the production of new neurons (by proliferation of neuronal progenitors that reside within the basal layers of the epithelium) is rapidly upregulated until the original state of the OE is restored (reviewed in Calof et al., 1996a). An in vitro correlate of this phenomenon is the observation that proliferation and generation of new ORNs by cultured OE neuronal progenitors is inhibited by the presence of large numbers of differentiated ORNs (Mumm et al., 1996). Such experiments have provided strong support for the idea that ORNs produce a signal that feeds back to inhibit production of new neurons by their own progenitors.

Because of their known actions in inhibiting cell growth, as well as neural induction, we have focused on signaling molecules of the transforming growth factor-β (TGF-β) superfamily as candidates for feedback inhibitors of neurogenesis (Shou et al., 1999, 2000). Here we provide in vitro and in vivo evidence that a recently identified member of this superfamily, growth and differentiation factor 11 (GDF11), acts as such a feedback inhibitory signal in the OE. Thus, one way in which the mammalian nervous system achieves proper neuron number during development is by negative autoregulation of neurogenesis. In the OE, GDF11 and its antagonist, follistatin, are critical regulators of this process.

Results

Expression of Gdf11 and Its Putative Receptors by Neurons and Neuronal Progenitors

The TGF-β superfamily, a large group of secreted proteins with widespread roles in development and tissue homeostasis, can be divided into two groups on the basis of similarities in structure and downstream signaling pathways: the TGF-β/activin group and the Dpp/bone morphogenetic protein (BMP) group (Newfeld et al., 1999). GDF11, a recently identified member of a
small subfamily of the TGF-β/activin group, came to our attention after a report showing its expression in the epithelium lining the nasal cavity (Nakashima et al., 1999). GDF11 is 90% identical in amino acid sequence to GDF8/myostatin, a factor that is expressed by muscle cells, inhibits proliferation of myoblasts in culture, and when absent causes mice to exhibit increased skeletal muscle mass (Lee and McPherron, 1999; McPherron et al., 1997; Taylor et al., 2001; Thomas et al., 2000). Because this is the same type of action we envisioned for feedback inhibition of neurogenesis, we performed in situ hybridization experiments to determine if Gdf11 and components of its signaling pathway have appropriate patterns of expression in the OE (Figure 1).

In mouse OE, Gdf11 expression is first evident at embryonic day 12.5 (E12.5), and continues to be expressed through adulthood (data not shown). At E14.5—the age at which the neuronal lineage is fully established and there is a high level of neurogenesis in the OE (Calof and Chikaraishi, 1989)—we found Gdf11 expression in the nasal mucosa to be confined to the olfactory (sensory) epithelium, with no expression in adjacent respiratory epithelium (Figure 1A). Gdf11 expression in facial muscle mass (Lee and McPherron, 1999; McPherron et al., 1997; Taylor et al., 2001; Thomas et al., 2000). Because this is the same type of action we envisioned for feedback inhibition of neurogenesis, we performed in situ hybridization experiments to determine if Gdf11 and components of its signaling pathway have appropriate patterns of expression in the OE (Figure 1).

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in developing head and trunk muscle (Figures 1B and 1C). At higher resolution, Gdf11 expression was seen to be confined to the basal two-thirds of the OE, the region that contains ORNs and their progenitors (Figure 1D). A similar expression pattern was observed for ActRIB and the type I TGF-β receptor Alk5, which other work has suggested are the likely ligand binding and signaling receptors for GDF11 (Figure 1D) (Federman et al., 2000; Lee and McPherron, 2001; McPherron et al., 1999). Interestingly, follistatin (Fst), a secreted antagonist of GDF11 (Gamer et al., 1999), is also expressed in OE, as well as its underlying stroma (Figure 1D).

Within OE proper reside both neuronal cells (ORNs and their progenitors) and two nonneuronal cell types, sustentacular cells (supporting cells found in a single, apical layer) and horizontal basal cells (keratin-expressing cells that lie immediately atop the basal lamina) (Figure 1E). The neuronal cells of the OE, which occupy the intervening layers, consist of ORNs and the three progenitor cell types of this neuronal lineage (Figure 1F). Thus, there are four distinct stages in the ORN lineage (reviewed in Calof et al., 2002): (1) a self-renewing stem cell, which gives rise to (2) neuronal progenitors that express the bHLH transcription factor MASH1. MASH1-expressing progenitors give rise to (3) immediate neuronal precursors (INPs), which express the bHLH transcription factor, Neurogenin1 (Ngn1). INPs divide to give rise to daughter cells that undergo terminal differentiation into (4) ORNs. Differentiated ORNs extend axon processes to the olfactory bulb of the brain, where they form synapses and express differentiation markers such as the neural cell adhesion molecule, NCAM.

To determine which OE cell types express Gdf11, we took advantage of Mash1−/− mice. Genetic studies have shown that Mash1 function is required for generation of ORNs (Guillemot et al., 1993). In Mash1−/− mice the ORN lineage is cut short at an early stage, as Mash1-expressing neuronal progenitors initially form, but then undergo apoptosis (Calof et al., 1996b; Cau et al., 1997; Murray et al., in press). Thus, the OE of Mash1−/− mice is markedly thinner than that of wild-types, and expression of Ngn1 and Ncam is drastically reduced since the epithelium lacks most ORNs and ORN progenitors (Figure 1G). When we examined the OE of Mash1−/− embryos, we found that Gdf11 expression was essentially absent (Figure 1G). Since sustentacular cells and horizontal basal cells are still present in Mash1−/− mice (Guillemot et al., 1993; Murray et al., in press), this indicates that the cells that express Gdf11 must be ORNs and ORN progenitors.

GDF11 Inhibits Development of the Progenitor Cells that Give Rise to Olfactory Receptor Neurons

An early indication that GDF11 was likely to exert a negative effect on OE neurogenesis came from pilot studies with GDF8 (which appears to activate the same signaling pathways as GDF11 [Federman et al., 2000; Lee and McPherron, 1999, 2001; McPherron et al., 1999; Oh and Li, 1997] and was initially more readily available). In these studies, we assessed effects of GDF8 on neuronal colony formation in vitro, an assay that gives a sensitive and quantitative overall picture of OE neurogenesis, but does not reveal the cellular stages at which effects occur (Mumm et al., 1996; Shou et al., 1999, 2000). We observed that GDF8 (10 ng/ml) treatment caused a 95 ± 5% decrease in the number of neuronal colonies that developed in these assays, compared to untreated controls.

With this information in hand, we turned to testing GDF11 itself, and to using assays that provide more detailed information about cellular targets of growth factor action, such as short-term OE explant cultures in which individual cells are easily identified and counted. Initially, OE explants were cultured for 22 hr in GDF11, with 3H-thymidine (3H-TdR) added for the final 2 hr to mark neuronal progenitors in S phase. As shown in Figure 2A, GDF11 caused a large decrease in the number of progenitors incorporating 3H-TdR, compared with untreated cultures. This indicates that GDF11 indeed has negative action on OE neurogenesis, and acts to inhibit proliferation of OE neuronal progenitor cells.

We have shown previously that a different TGF-β superfamily ligand, BMP4, has an antineurogenic action in the OE neuronal lineage that is exerted on MASH1-expressing neuronal progenitors. BMP4 binding to these cells targets preexisting MASH1 protein for rapid degradation via the proteasome pathway, resulting in cessation of proliferation, blockade of the ORN developmental pathway at the MASH1+ cell stage, and eventually, cell death (Shou et al., 1999). Interestingly, GDF11 does not act via this mechanism: when we grew OE explant cultures in the presence or absence of GDF11 for 8 hr in vitro—a time when MASH1 expression is maximal under...
normal culture conditions—we saw no effect of GDF11 on Mash1 expression (Figure 2B). BMP4 treatment for this same time period, in contrast, results in almost complete loss of Mash1 expression [Shou et al., 1999]. Not only does GDF11 fail to target Mash1 for degradation, it also has no antiproliferative effect on Mash1-expressing progenitors: the percentage of Mash1+ cells incorporating ³H-TdR is unchanged by treatment with GDF11 (Figure 2C). Thus, GDF11 has a strong negative effect on neuronal progenitors, but not by acting on those which express Mash1.

The progeny of Mash1-expressing cells are INPs, the direct progenitors of ORNs (Figure 1F) and the most abundant progenitor cell type in OE explant cultures (Calof and Chikaraishi, 1989; DeHamer et al., 1994). Fibroblast growth factor 2 (FGF2) stimulates INP divisions, and essentially all of the increase it brings about in ³H-TdR incorporation by cells in OE explant cultures at 48 hr in vitro can be ascribed to an effect on INPs (DeHamer et al., 1994; Shou et al., 2000). We reasoned that if GDF11 exerts its inhibitory effect on INPs, it might abrogate the effect of FGF2. Indeed, as shown in Figure 2D, treatment with GDF11 completely abolishes the stimulatory effect of FGF2 on INPs, strongly suggesting that GDF11 inhibits INP divisions.

To show such an action directly, we needed a means of marking INPs in culture. Since INPs express Ngn1, we used a transgenic mouse line, TgN1-2G, in which GFP is expressed under the control of Ngn1 regulatory elements (Gowan et al., 2001). To verify that GFP marks the correct cells in the OE, we crossed the TgN1-2G allele onto a Mash1−/− background (these mice lack Ngn1-expressing cells in most of the OE [Figure 1G] [see also Cau et al., 1997]) and observed that GFP expression in the OE indeed disappeared (Figure 3A). We also verified that GFP and Mash1 are expressed in distinct cells in explant cultures of TgN1-2G OE (Figure 3B), as expected if GFP marks INPs.

With this confirmation, we then tested directly the effect of GDF11 on OE explants cultured from TgN1-2G embryos. As shown in Figure 3C, GDF11 treatment results in a 3-fold decrease in the number of GFP-expressing cells that develop over the course of 22 hr, in a manner that is completely blocked by follistatin. Because GDF11 has no effect on proliferation of the Mash1-expressing cells that give rise to INPs (Figures 2B and 2C), we concluded that the decrease in GFP-expressing cells caused by GDF11 reflects a loss, or failure to expand, of existing INPs.

**GDF11 Treatment Leads to Cell Cycle Arrest in INPs**

The findings above indicated that GDF11 blocks the ORN developmental pathway at the INP stage, but left unclear the mechanism. To investigate this, we cultured OE explants from TgN1-2G embryos for 8, 16, or 24 hr, with and without added GDF11 (Figure 4A). Under control conditions, total cell number increased linearly between 8 and 24 hr, reflecting proliferation of both Mash1+ progenitors and INPs, and the generation of ORNs. In GDF11-treated cultures, however, there was no increase in total cell number. Similarly, in control cultures the number of GFP+ cells (presumed INPs) increased steeply from 8 to 16 hr and then began to plateau, reflecting division of INPs followed by their generation of terminally differentiated ORNs (Figure 4B) (Calof and Chikaraishi, 1989; DeHamer et al., 1994). In marked contrast, no increase in GFP+ cell number occurred in GDF11-treated cultures, although similar numbers of INPs were present initially whether or not GDF11 had been applied (Figure 4B). As expected, Mash1-expressing progenitors, which normally decline in number during culture (Gordon et al., 1995), were unaffected by GDF11 treatment at any time point tested (Figure 4C).

The failure of INP numbers to increase in GDF11-treated cultures might be due to decreased INP proliferation, but could also be caused by an effect on INP survival. To test this, we performed TUNEL assays on OE explants grown for 19 hr in the presence of GDF11 (Holcomb et al., 1995). The fraction of INPs (GFP+ cells) undergoing apoptosis was not significantly different in GDF11-treated cultures than in untreated controls (Figure 4D). This result supports the view that, in the presence of GDF11, INPs remain alive, but no longer progress through the cell cycle and therefore no longer generate ORNs.

If GDF11 acts by causing cell cycle arrest, then its effect might be reversible. To test this, we grew TgN1-2G−/− explants for 12 hr in GDF11, and then removed GDF11 from half of the cultures. Explants were maintained for an additional 18 hr, with ³H-TdR added for the final 6 hr to label cells in S phase. In cultures from which GDF11 had been removed, more than twice as
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Figure 4. GDF11 Reversibly Inhibits Division of INPs

(A–C) OE explants from TgN1-2G/H11001/H11002 embryos were grown with or without GDF11 (20 ng/ml) and fixed at indicated times. Total migratory cells (A), GFP+ cells (B), and MASH1+ cells (C) surrounding individual explants were counted. (A) Error bars, SD; p < 0.05 for t = 24 hr; (B) Error bars, SD; p < 0.05 for t = 16 hr and t = 24 hr (Student’s t test); (C) Error bars, SEM.

(D) GDF11 does not promote apoptosis of INPs. OE explants isolated from TgN1-2G/H11001/H11002 embryos were cultured with or without GDF11 (20 ng/ml) for 19 hr, processed for TUNEL, and the percentage of GFP+ cells that were TUNEL+ determined. Error bars, SEM.

(E and F) GDF11’s effect on INPs is reversible. OE explants were treated with 20 ng/ml GDF11 for 12 hr, then GDF11 was removed and cultures re-fed with medium containing follistatin (100 ng/ml; “GDF11 to Fol”) or 20 ng/ml GDF11 (“GDF11 to GDF11”). Control cultures had no factor added for the first 12 hr, then were switched to medium containing 100 ng/ml follistatin. Total GFP+ cells and “H-TdR” cells surrounding each explant were counted. Error bars were calculated from the square root of the sum of the squares of fractional errors for control and experimental values. p < 0.005 for (E) and (F); Student’s t test.

Many GFP+ INPs were present at the end of the culture period (Figure 4E). Moreover, these cells were capable of dividing, as shown by incorporation of “H-TdR (Figure 4F). These results show that INPs are still present and viable in GDF11-treated cultures, but are reversibly arrested in the cell cycle.

We speculated that GDF11-induced cell cycle arrest was likely to occur in G1, a crucial phase of cell cycle control for many kinds of progenitors. The cyclin-dependent kinase inhibitor p27Kip1 has been implicated as a mediator of G1 phase cell cycle arrest induced by TGF-β (e.g., Polyak et al., 1994). Since the type I TGF-β receptor, ALK5, is also the likely signaling receptor for GDF11 (Federman et al., 2000), and since p27Kip1 is known to be involved in regulating proliferation of neuronal progenitors (Chen and Segil, 1999; Dyer and Cepko, 2001; Levine et al., 2000; Miyazawa et al., 2000), we hypothesized that GDF11-induced cell cycle arrest in INPs might be accompanied by increased expression of p27Kip1. To test this idea, we grew OE explants for 14 hr in the presence or absence of GDF11 and then processed the cultures with an antibody to p27Kip1 (Figure 5). Indeed, in OE explants treated with GDF11, the percentage of migratory neuronal cells expressing detectable levels of p27Kip1 immunoreactivity was 10-fold higher than in untreated controls (Figure 5B), and the majority of these p27+ cells were NCAM-negative (i.e., neuronal progenitors) calculated. Error bars, SEM; p < 0.001, Student’s t test.

Figure 5. p27Kip1 Levels Are Increased in GDF11-Treated Neuronal Progenitor Cells

OE explants were cultured with or without GDF11 (20 ng/ml) for 14 hr and processed for p27Kip1 and NCAM immunoreactivity.

(A) Many migratory cells in GDF11-treated cultures express detectable levels p27Kip1, almost none do in control (Ctrl; untreated) cultures (white arrow indicates a cell with low-level expression).

(E and F) GDF11’s effect on INPs is reversible. OE explants isolated from TgN1-2G/H11001/H11002 embryos were cultured with or without GDF11 (20 ng/ml) for 19 hr, processed for TUNEL, and the percentage of GFP+ cells that were TUNEL+ determined. Error bars, SEM.

(F) Each p27Kip1 cell in (B) was evaluated for NCAM immunoreactivity,

Consistent with this expectation, the gross phenotype

Increased Neurogenesis in Mice Lacking Functional Gdf11

To determine if endogenous GDF11 regulates OE neurogenesis in vivo, we disrupted the mouse Gdf11 gene, inserting a neo cassette into exon 3, which encodes the mature peptide (Figure 6A). This allele (referred to as Gdf11tm2) is expected to be functionally null, as it encodes an aberrant transcript in which codons for four of the seven cysteines critical for proper structure and function of TGF-β are absent (Schuefler et al., 1999).
Figure 6. Disruption of Neurogenesis in Mice with Loss- or Gain-of-Function of Gdf11

(A) Analysis of Gdf11	m2/tm2 mice. (1) Gdf11 gene (wild-type allele). Lightly shaded box, exon 3; P1 and P2, locations of forward and reverse primers used for RT-PCR analysis in (6). “A,” Apal; “B,” BamH I; “H,” HindIII; “N,” Nodl; “S,” Sall; “X,” Xbal. (2) Targeting vector. NEO, PGKneoA cassette; TK, MC1-thymidine kinase cassette. (3) Targeted allele. Double-headed arrows indicate expected lengths of HindIII fragments for wild-type and targeted alleles detected using probe indicated in (1). A HindIII site is introduced into the targeted allele by the PGKneoA cassette. (4) Wild-type and Gdf11	m1022 littermates at E15.5; note lack of tail in Gdf11	m1022 embryo (asterisk). (5) Southern analysis of HindIII-digested DNAs from wild-type and targeted alleles de-
incorporating cells (see above). We also examined Mash1 expression in the OE, both by in situ hybridization and with a monoclonal antibody to Mash1, and failed to detect any significant change between Gdf11\textsuperscript{tm2/tm2} animals and wild-types (there were 258 ± 58 [SD] Mash1-immunopositive cells per millimeter in wild-type OE, versus 286 ± 32 [SD] Mash1-immunopositive cells per millimeter in Gdf11\textsuperscript{tm2/tm2} OE). Together, these results indicate that the overall increase in proliferating cells in Gdf11\textsuperscript{tm2/tm2} OE is due specifically to an increase in the number of INPs. That these supernumerary progenitors go on to give rise to neurons was demonstrated by an increase in Ncam-expressing cells in Gdf11\textsuperscript{tm2/tm2} OE: the Ncam-expressing cell layer is thicker by 20% (9 μm, about the diameter of one ORN) in Gdf11\textsuperscript{tm2/tm2} OE than in wild-types. (The average thickness of the Ncam\textsuperscript{+} layer of the OE was measured in sections hybridized with the Ncam probe, as illustrated in Figure 6B; values obtained were 33.05 ± 1.04 μm [SEM] for wild-type OE, versus 42.49 ± 0.98 [SEM] for Gdf11\textsuperscript{tm2/tm2} OE [p < 0.05, Student’s t test]). Thus, not only is proliferation of INPs increased in Gdf11\textsuperscript{tm2/tm2} animals, so is production of differentiated neurons.

**Decreased Neurogenesis in Mice Lacking Follistatin, a GDF11 Antagonist**

If GDF11 truly functions as an endogenous negative regulator of neurogenesis in the OE, then an increase in GDF11 activity would be expected to result in a decrease in neurogenesis, to below normal levels. Follistatin has been shown to antagonize GDF11 activity in *Xenopus* animal cap assays (Gamer et al., 1999), and in OE cultures, follistatin is able to antagonize the antineurogenic effect of GDF11 on INPs (Figure 3). Since Fst mRNA is also expressed within the OE (Figure 1D), we reasoned that mice lacking a functional Fst gene might show evidence of increased GDF11 activity in the OE. This idea was tested by examining the OE of Fst\textsuperscript{−/−} embryos (Fst\textsuperscript{−/−} mice die at birth [Matzuk et al., 1995]), using markers for OE neuronal cells and BrdU incorporation to detect proliferating progenitors. The data are shown in Figure 6C. In situ hybridization for Ngn1 and Ncam showed large decreases in expression of both markers, indicating that production of both INPs and ORNs is profoundly decreased in Fst\textsuperscript{−/−} OE. Strikingly, we also observed a 37% decrease in the number of BrdU-incorporating cells and a 38% decrease in OE thickness in Fst\textsuperscript{−/−} animals. These results indicate that the action of follistatin is required for normal levels of neurogenesis in the OE. Since follistatin by itself has no effect on neurogenesis when tested in OE explants (Figure 3), these findings also suggest that the decrease of neurogenesis in Fst\textsuperscript{−/−} animals is the result of increased activity of a molecule antagonized by follistatin, such as GDF11.

**Discussion**

**Negative Autoregulation of Neurogenesis by Endogenous Signaling Molecules**

How tissues reach and maintain their appropriate sizes has been the subject of speculation for many years. Almost 40 years ago, Bullough put forward the hypothesis that tissues produce growth-inhibitory signals, called "chalones," the local concentrations of which directly reflect the mass of the tissue in which they are produced (Bullough, 1965). Such signals were proposed to halt cell proliferation when appropriate tissue size had been reached, thereby maintaining the cell number appropriate for tissue function. The discovery of GDF8 (myostatin), a signaling molecule of the TGF-β superfamily which is both made by developing muscle cells and inhibits their proliferation, has validated this idea (Lee and McPherron, 1999).

For several years, we have sought to identify the molecular signal(s) that mediate feedback inhibition of neurogenesis in the OE. Recently, we suggested that BMPs, and in particular BMP4, may provide such a signal. BMP4 is expressed in OE and, in vitro, blocks the ORN lineage at the MASH1\textsuperscript{+} progenitor cell stage (Shou et al., 1999). Although these observations make BMP4 a plausible candidate for a feedback inhibitor of neurogenesis, the additional facts that (1) Bmp4 is expressed not only in OE proper, but also elsewhere in the nasal region (e.g., in OE stroma); and (2) BMP4 effects on the ORN lineage are complex, with BMP4 actually promoting OE neurogenesis at low concentration by supporting ORN survival (Shou et al., 2000), prompted our search for other TGF-β-s that might be better candidates for the endogenous negative growth signal. This led us to investigate GDF11.

The data presented here demonstrate that GDF11 is a critical endogenous inhibitor of OE neurogenesis. Its effects in vitro are directed at a specific stage of transit-amplifying progenitors, the Ngn1-expressing INPs. GDF11 does not drive INPs into apoptosis, as BMPs do with some neuronal progenitors (e.g., Shou et al., 1999), nor does it reduce progenitor cell number by promoting neuronal differentiation, another mechanism by which BMPs have been reported to act (e.g., Li et al., 1998). Instead, GDF11 reversibly blocks INP divisions (Figure 4), and this is associated with increased expression of the cyclin-dependent kinase inhibitor, p27\textsuperscript{kip1} (Figure 5). In vivo, lack of Gdf11 function results in an increase in proliferating, Ngn1-expressing INPs and an approximate 20% increase in neuron number within the epithelium (Figure 6). Conversely, in mice lacking a functional follistatin gene (Matzuk et al., 1995), which encodes a secreted GDF11 antagonist (Gamer et al., 1999), there is a substantial decrease in OE neurogenesis. This is evident as a decrease in both OE thickness and the number of proliferating progenitors within the OE, as well as by decreases in Ngn1\textsuperscript{−} INPs and Ncam-expressing ORNs (Figure 6). Together, these observations demonstrate that the GDF11 and follistatin are both of crucial importance in regulating OE neurogenesis, and suggest that follistatin’s role in vivo, at least in part, is to modulate the activity of endogenous GDF11. (Since follistatin also binds with high affinity to activin [Schneyer et al., 1994] and antagonizes activin’s biological activities [Nakamura et al., 1990], it remains possible that endogenous activin [Feijen et al., 1994] may also interact with follistatin in modulating OE neurogenesis. This possibility is currently under investigation in our laboratory.)

It is not known if the CNS controls neuron number using a mechanism similar to the feedback inhibitory mechanism we have proposed for OE. An important
part of determining whether such a mechanism might operate will be to test likely signaling molecules for their potential role(s) as autocrine-negative regulators of neurogenesis. Significantly, both GDF11 and follistatin show widespread expression in the CNS (data not shown) (Feijen et al., 1994; Nakashima et al., 1999; Roberts and Barth, 1994), including regions such as the dentate gyrus of the hippocampus, the external granule layer of the cerebellum, and neural retina, where neurogenesis is known to be tightly controlled by cell interactions (e.g., Parent et al., 1997; Sloviter et al., 1996). Given these facts, it is reasonable to hypothesize that GDF11 and follistatin may be important players in a process of feedback inhibition of neurogenesis that could act to regulate neuron number during development (and, potentially, regeneration) of the CNS.

Regulation of Neuron Number by GDF11

Although GDF11 clearly acts as a negative regulator of neurogenesis in the OE, we found no evidence that it affects neuronal cell survival or fate. All neuronal cell types are still present in the OE of Gdf11 tm2tm2 mice; only their numbers, and consequently the overall thickness of the OE, are altered (Figure 6). Thus, GDF11, like the chalones proposed by Bullough (1965), appears to act in an autocrine fashion as a dynamic negative regulator of OE tissue size.

Data from in vitro experiments suggest that GDF11 may induce cell cycle arrest of INPs by increasing the expression of the cyclin-dependent kinase inhibitor, p27Kip1 (Figure 5). In fact, the OE of Gdf11 tm2tm2 mice has been reported to contain an increased number of BrdU-incorporating cells (Legrier et al., 2001), raising the possibility that p27Kip1 is a major downstream target of GDF11 in vivo. In this light, it may be significant that the percentage increase in thickness of the OE in Gdf11 tm2tm2 mice (~22%) is similar to the increase in brain size (~18%) observed in mice with targeted inactivation of the p27Kip1 gene (Fero et al., 1996). In considering the importance of this effect, it is notable that such an increase in size in a neural tissue can profoundly disrupt function. For example, the modest increase in hair cell number in the inner ear of p27Kip1 mice (23% increase in inner hair cells; 36% in outer hair cells) results in profound hearing impairment (Chen and Segil, 1999). Such findings emphasize how important control of neuron number is to nervous system function.

Is GDF11 Important for Neuronal Regeneration?

The OE is one of the few regions of the mammalian nervous system with the capacity for true neuronal regeneration, and the action of GDF11 on INPs may be of special significance in regulating the temporal dynamics of this process. INPs function as transit-amplifying cells in the ORN lineage, proliferating in response to extrinsic cues but remaining committed to a neuronal (ORN) fate (DeHamer et al., 1994). In vivo, death of ORNs provides such a cue, causing INP proliferation to increase rapidly and remain elevated until neuron number is restored (reviewed in Calof et al., 1996a). Thus, in the OE, as in many regenerating tissues, transit-amplifying cells, by rapidly altering their proliferation in response to extrinsic cues, provide the capacity for rapid changes in the sizes of differentiated cell populations in response to changing environmental demands (Hall and Watt, 1989; Potten and Loeffler, 1990). By inducing a reversible state of growth inhibition, GDF11 may play an important role in maintaining the INP population in an appropriate state to respond rapidly to environmental signals. GDF11’s ability to induce p27Kip1 is likely to be of importance in this process, as p27Kip1 has been shown recently to be important in determining numbers of transit-amplifying progenitors elsewhere in the nervous system (Doetsch et al., 2002).

Parallels between Neurogenesis and Myogenesis

A striking finding of the present study is the degree to which mechanisms of feedback regulation of tissue size appear to have been conserved between neuronal and muscle lineages (Figure 7). In muscle, not only does GDF8 exert the same sort of growth inhibitory effect as GDF11 in the OE (Lee and McPherron, 1999), GDF8 also appears to utilize an analogous mechanism: inducing G1 phase arrest in early myoblasts (Thomas et al., 2000), which are FGF-stimulated transit-amplifying cells similar to INPs (Clegg et al., 1997). Furthermore, GDF8-induced cell cycle arrest is characterized by an increase in expression of p21Cip1/Waf1 (Thomas et al., 2000), a homolog of p27Kip1 that acts similarly to cause cell cycle arrest. Interestingly, such mechanisms may be utilized not only in vertebrate tissues, but also in invertebrates: the recent description of Drosophila GDF-11/GDF-8 and activin homologs, which are highly expressed in muscle, glial, and neuronal progenitors (Lo and Frasch, 1999), suggests that these TGF-βs may function as negative regulators of cell division in the same Drosophila tissues as those in which they act in vertebrates. Altogether, these results suggest that fundamental mechanisms of tissue size regulation are evolutionarily ancient and thus are likely to be of great importance as control mechanisms during development and regeneration.

![Figure 7. Parallels between the Regulation of Myogenesis and Neurogenesis](image-url)
Experimental Procedures

Materials

Recombinant human GDF8 and GDF11 were from Genetics Institute/Wyeth. GDF11 was used as conditioned medium collected from CHO cells stably transfected with human Gdf11 cDNA (GenBank AF1019080); GDF11 concentration was quantified by immuno blotting. Follistatin was obtained through the National Hormone & Pituitary Program and A.F. Parlow, Harbor-UCLA Medical Center (Torrance, CA). Recombinant human FGF2 (157 aa form) was from R&D Systems.

Animals

Day of vaginal plug detection was designated day 0.5 of embryonic development. Mash1+/− mice were maintained on a CD-1 (Charles River) background, where the OE phenotype is fully penetrant (our observations and Cau et al. [1997]). TgNt1-2G transgenic mice were maintained as homozygotes on a CD-1 background; to obtain OE for tissue culture, TgNt1-2G+/− males were mated with CD-1 females and the resulting offspring using. Fst+/− animals were maintained on an inbred C57BL/6J background. Fst−/− animals were genotyped using primers to the inserted human Hprt sequences (targeted allele: forward primer, 5′-GGCAAAGGTGATGACTTGGAGG-3′; reverse primer, 5′-CCAGTTTCTAATGAGCAACAGCT-3′) and sequences within exon 2 and 3 of Fst (wild-type allele: forward primer, 5′-CTGACCGACCTGTGAGCAGCGA-3′; reverse primer, 5′-CACATTCGTTGGCCTAGTT3′). The Fst wild-type allele was detected as an ~700 bp fragment and the targeted allele as a ~650 bp fragment.

Mice deficient in Gdf11 (Gdf11tm2 mice) were generated by gene targeting. Gdf11 clones were isolated from a 129/Sv mouse genomic DNA λ phage library (Stratagene). The targeting construct (Figure 6A) was generated by inserting a neomycin resistance gene under the control of the PGK promoter (PGKneoAP) into the Apal site in the third coding exon. The 5′ and 3′ flanks consists, respectively, of 11.1 and 6.0 kb Apal fragments. An MC1-thymidine kinase cassette (MC1tkPα) is downstream of the 3′ flank. Plasmid sequences were released by digestion with Sall, and the targeting vector electroporated into RW-4 ES cells (Genome Systems). Correctly targeted ES clones were identified by Southern blot analysis, and two independent clones introduced into the mouse germline by blastocyst injection (Pogran et al., 1994). Heterozygous mice appeared normal and were intercrossed to obtain homozygous mutant mice. Gdf11tm2/ tm2 littermates used in this study were generated by intercrossing autoradiography as described previously (DeHamer et al., 1994; Shou et al., 1999). Digoxigenin-labeled cRNA probes were: 1.2 kb mouse Gdf11 partial cDNA (bp 229–1218 of coding region; Genbank #AH069682) plus ~500 bp of 3′ non-coding sequence; bases 1201–1835 of mouse Gdf8 3′ UTR (Genbank #NM010834); 437 bp mouse Akt5 (87–511 bp of Genbank #NM009307); 308 bp mouse ActRlb (119–427 bp of Genbank #M84120); 770 bp partial mouse fst cDNA (Albano et al., 1994); Ngn1 (1.2 kb fragment of rat Ngn1 gene [Ma et al., 1996]); and Mash1 (2.0 kb fragment of mouse Mash1 gene including coding region and 3′ UTR [Guillemot and Joyner, 1993]). Hyridization was detected using alkaline phosphatase-conjugated sheep anti-digoxigenin Fab fragments, followed by BCIP/NBT according to manufacturer’s instructions (Roche).

For quantification of BrdU- and MASH1-immunopositive cell numbers, as well as overall OE thickness and thickness of Ncam-expressing cell layers, multiple adjacent fields of OE lining the nasal septum (chosen because this is an uncurved structure with an OE lining of regular thickness) were evaluated at 400× magnification. In comparing wild-type and transgenic animals, sections from similar levels along the dorsal-ventral axis were chosen for analysis in all cases.

To detect p27Kip1 cultures were fixed and processed as described for Mash1 immunostaining (Shou et al., 1999); the primary antibody (monoclonal anti-p27Kip1; clone 57; BD Transduction Laboratories) was detected using rabbit anti-mouse IgG1 (Harlan) followed by AlexaFluor 594-conjugated goat anti-rabbit IgG (Molecular Probes). Confirmation that this antibody recognizes authentic p27Kip1 in OE cultures was obtained by immunoblotting (data not shown). To quantify p27Kip1 immunoreactivity, individual cells were imaged under rhodamine optics with a 40× oil objective (Zeiss) using a cooled CCD digital camera (Diagnostics Instruments SP100, 1315 × 1035 pixel resolution). Raw data files were imported to NIH ImageJ v.1.28 (r142); and total migratory cells and cells with mean fluorescence intensities ≥5(“p27Kip1” cells”) counted (mean background intensity for controls ~3.7 ± 0.2; for GDF11-treated culture ~3.9 ± 0.2). TUNEL staining of cultures was performed as described (Holcomb et al., 1998), using biotin-16-dUTP detected with Texas Red-conjugated streptavidin (Molecular Probes).

Primary OE Cultures

OE explant cultures were prepared as described previously (DeHammer et al., 1994; Shou et al., 1999) and grown in vitro for the indicated times. H-thymidine (H-TdR; 60–80 Ci/mmol, 1 mCi/ml, ICN) was applied at the following concentrations: 1.5 μCi/ml for the final 8 hr in explants cultured for 22 hr, 0.5 μCi/ml for the final 2 hr in explants cultured for 8 hr, and 0.1 μCi/ml for the final 24 hr in explants cultured for 48 hr. Cultures were fixed and processed for autoradiography as described previously (DeHammer et al., 1994; Shou et al., 1999). For comparison of labeled migratory cell numbers among different explants in a given experiment, total numbers of cells surrounding each explant were counted and the size of the explant measured using NIH Image. Because explants are irregular in area, the number of H-TdR+ cells for each explant was normalized to an area value of 15,000 μm2, the average size of explants in these cultures (cf. DeHammer et al., 1994).

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