

vertebrates.  $\text{Ca}^{2+}$  signaling appears to drive dorsoventral axis patterning of vertebrates, whereas such noncanonical Wnt signaling (that is, signaling not mediated by  $\beta$ -catenin) regulates focal adhesion kinase activity and cell motility in *Drosophila* (15). Details about substrates of PKC (16), CamKII, and calcineurin specifically involved in the downstream events of Wnt- $\text{Ca}^{2+}$  signaling are few. The transcription factor (TF) NF-AT is both a substrate for calcineurin and a regulator of ventral cell fate (14), providing one carefully detailed example. Targets of the Wnt- $\text{Ca}^{2+}$  pathway appear to cross-talk to the Wnt- $\beta$ -catenin pathway at multiple points (17). The fact that PKC appears to participate not only in the Wnt- $\text{Ca}^{2+}$  pathway but also in the Wnt- $\beta$ -catenin (17) and perhaps in the Wnt-PCP pathways suggests that cellular context and temporal and spatial considerations must be highlighted in our quest to fully understand Wnt- $\text{Ca}^{2+}$  signaling.

We have implicated the G protein transducer 2 ( $G_{12}$ ), cGMP PDE, and intracellular cGMP in Wnt- $\text{Ca}^{2+}$  signaling (7). Each of these signaling elements is well known in the visual excitation pathway of both invertebrates and vertebrates (18). Disruption of the  $G_{12} \rightarrow \text{PDE} \rightarrow \text{cGMP}$  pathway in response to Wnt activation blocks primitive endoderm formation in mouse F9 cells and normal early development in zebrafish embryos (7). There may be cross-talk among the Wnt signaling pathways mediated by cGMP. Inhibitors of cGMP PDEs are reported to inhibit the Wnt- $\beta$ -catenin path-

way by increasing intracellular concentrations of cGMP, activating cGMP-dependent protein kinase (which phosphorylates  $\beta$ -catenin), reducing  $\beta$ -catenin concentrations, and promoting apoptosis (19). The PDE inhibitor Exisulind has been proposed as a lead compound for anticancer therapies (20).

Understanding the Wnt- $\text{Ca}^{2+}$ -cGMP pathway and its full biological role may yield rich dividends in several areas. Genes regulated by the Wnt- $\beta$ -catenin pathway have been described (21), whereas gene profiling for the Wnt- $\text{Ca}^{2+}$ -cyclic GMP pathway has not been reported. Several early lines of evidence suggest that the Wnt- $\text{Ca}^{2+}$ -cGMP pathway may antagonize the Wnt- $\beta$ -catenin pathway (17), which has many linkages to human cancer. Unknown is the extent to which the Wnt- $\text{Ca}^{2+}$ -cGMP pathway may participate in the regulation of the PCP function of Wnts. Because Wnts can interact with more than one Frizzled, detailed analysis of individual Frizzleds may yield new insights into Frizzled-specific pathways of signaling and gene expression. The role of low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) as coreceptors with Frizzled in the Wnt- $\beta$ -catenin pathway raises the question of a possible similar role for LRP proteins in Wnt- $\text{Ca}^{2+}$  signaling. Finally, the macromolecular complexes involved in Wnt-Frizzled signaling are tantalizing targets for analysis. Scaffolds such as Axin, as well as A kinase anchor proteins (22), will likely provide explanations for the exquisite temporal and spatial responses we find for many GPCR-mediated pathways, including Wnt sig-

aling. Given the apparent function of primary elements of the visual pathway in the regulation of embryonic development, we can also imagine other roles for Wnts and Frizzleds outside of their critical roles in early development.

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#### VIEWPOINT

## Myocyte Adrenoceptor Signaling Pathways

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Adrenoceptors (ARs), members of the G protein-coupled receptor superfamily, form the interface between the sympathetic nervous system and the cardiovascular system, with integral roles in the rapid regulation of myocardial function. However, in heart failure, chronic catecholamine stimulation of adrenoceptors has been linked to pathologic cardiac remodeling, including myocyte apoptosis and hypertrophy. In cardiac myocytes, activation of AR subtypes results in coupling to different G proteins and induction of specific signaling pathways, which is partly regulated by the subtype-specific distribution of receptors in plasma membrane compartments containing distinct complexes of signaling molecules. The Connections Maps of the Adrenergic and Myocyte Adrenergic Signaling Pathways bring into focus the specific signaling pathways of individual AR subtypes and their relevant functions in vivo.

Adrenoceptors (ARs) belong to the G protein-coupled receptor (GPCR) superfamily. ARs connect the sympathetic nervous system and the cardiovascular system, playing an integral

role in the rapid regulation of myocardial function (1). However, in heart failure, chronic catecholamine stimulation of ARs may contribute to pathologic cardiac remodeling, including myocyte apoptosis and hypertrophy (2–5) (Fig. 1).

Cardiac myocytes express at least six types of ARs, including three types of  $\beta$ ARs ( $\beta_1$ ,  $\beta_2$ , and  $\beta_3$ ) and three types of  $\alpha_1$ ARs

( $\alpha_{1A}$ ,  $\alpha_{1B}$ , and  $\alpha_{1D}$ ) (1). Myocytes do not appear to express  $\alpha_2$ ARs in most species. On the basis of receptor number,  $\beta_1$ AR is generally the predominant receptor subtype in cardiac myocytes, although the ratio between  $\beta_1$  and  $\beta_2$  receptors varies among species, and  $\beta_2$ AR signaling is species-specific and dependent on age and developmental stage.

Both  $\beta_1$  and  $\beta_2$ ARs couple to the stimulatory  $G_s$  protein, which leads to activation of adenylyl cyclase and production of adenosine 3',5' monophosphate (cAMP). The cAMP-dependent protein kinase A (PKA) phosphorylates various substrates, including the L-type  $\text{Ca}^{2+}$  channel, which increases  $\text{Ca}^{2+}$  entry into cells; phospholamban, which accelerates  $\text{Ca}^{2+}$  sequestration into the sarcoplasmic reticulum resulting in accelerated cardiac relaxation (6); and troponin I and C proteins, which reduce myofilament sensitivity to  $\text{Ca}^{2+}$  (6). The ryanodine receptor is

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also a substrate for PKA, and ryanodine receptor phosphorylation has been observed in the failing human heart and in animal models of heart failure (7, 8). In vivo and in vitro assays show that the  $\beta_1$ AR plays the predominant role in modulating the heart rate and the force of contraction in the mouse (9, 10).

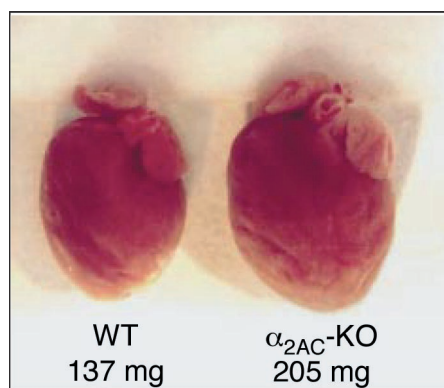
In contrast, activated  $\beta_2$ AR can couple to both  $G_s$  and  $G_i$  (G protein subunits that can inhibit adenylyl cyclases) in cardiac myocytes. In adult mouse cardiac myocytes, stimulation of the  $\beta_2$ AR selectively modulates the activity of adjacent L-type  $Ca^{2+}$  channels (6). However, in myocytes treated with pertussis toxin to disrupt  $G_i$  function, stimulation of  $\beta_2$ ARs leads to a more generalized activation of L-type  $Ca^{2+}$  channels, producing a robust increase in phospholamban phosphorylation, similar to that observed after stimulation of the  $\beta_1$ AR, which accelerates cardiac relaxation rate (6). In mouse neonatal cardiac myocytes, activated  $\beta_2$ ARs sequentially couple to both  $G_s$  and  $G_i$ , which induces a small increase followed by a sustained decrease in myocyte contraction rate (10). Stimulation of  $\beta_2$ ARs leads to a small cAMP accumulation; however, inhibition of PKA does not affect the magnitude of the myocyte contraction rate increase or decrease (10).

$\beta_3$ ARs have been detected in both human and murine hearts, although at lower levels than  $\beta_1$  and  $\beta_2$ ARs, and the physiologic role of  $\beta_3$ AR in regulating cardiac function is poorly understood. Stimulation of  $\beta_3$ ARs in human ventricular muscle reduces the strength of myocyte contraction by a  $G_i$ -dependent mechanism (11). Similarly, stimulation of  $\beta_3$ ARs results in a  $G_i$ -dependent decrease in the spontaneous contraction rate of cultured neonatal cardiac myocytes from  $\beta_1$ - $\beta_2$ AR knockout (KO) mice (10). However, stimulation of human  $\beta_3$ AR overexpressed in hearts of transgenic mice leads to an increase in the strength of contraction (12).

Recent studies have revealed an additional role of  $\beta$ AR stimulation in animal hearts. Chronic stimulation of the  $\beta_1$ AR induces myocyte apoptosis, although the signaling pathway is controversial. In vitro studies suggest that this process requires a PKA-independent activation of intracellular  $Ca^{2+}$  through L-type  $Ca^{2+}$  channels, which leads to activation of calmodulin kinase II in adult cardiac myocytes (13). However, this  $\beta_1$ AR-stimulated pro-apoptotic effect appears to be blocked by PKA inhibition in adult rat myocytes (14). In contrast, activation of  $\beta_2$ ARs has an anti-apoptotic effect, which is mediated by the  $\beta\gamma$  subunits of  $G_i$  in both rat and mouse adult cardiac myocytes (2, 15). The role of specific downstream effectors in  $G_i$  signaling remains less clear. Studies suggest that phosphatidylinositol 3-kinase (PI3K) can be activated by the  $\beta\gamma$  subunits of  $G_i$ , which leads to activation of the protein kinase B (Akt) pathway that confers the  $G_i$ -mediated

protection against apoptosis in adult mouse cardiac myocytes (15). Stimulation of  $\beta_2$ AR also leads to activation of the p38 mitogen-activated protein kinase (MAPK), although the role of p38 in mediating the anti-apoptotic effect of  $\beta_2$ AR stimulation is not clear (2). Interestingly, inhibiting the  $G_i$  pathway turns  $\beta_2$ AR signaling from anti-apoptotic into pro-apoptotic in mouse adult myocytes, suggesting that  $\beta_2$ AR to  $G_s$  signaling can cause myocyte apoptosis when coupling of  $\beta_2$ AR to  $G_i$  is inhibited (15).

Messenger RNA (mRNA) for all three  $\alpha_1$ AR subtypes have been detected in mammalian cardiac myocytes; however,  $\alpha_{1A}$ AR and  $\alpha_{1B}$ AR appear to play the major roles in regulating myocyte function (16–20).  $\alpha_1$ ARs couple to a broad spectrum of signaling pathways [including phospholipases C, D, and  $A_2$ ; protein kinase C (PKC);  $Ca^{2+}$  channels; and MAPK] by activating G proteins in the  $G_q$  family, as well as pertussis toxin-sensitive G proteins (17). The specific signaling pathways activated are influenced by receptor



**Fig. 1.** Hearts from wild-type (left) and  $\alpha_2AC$  double-knockout (right) mice.  $\alpha_2A$  and  $\alpha_2C$  adrenoceptors regulate catecholamine release from sympathetic nerve terminals. Mice with both  $\alpha_2$  receptors knocked out have elevated catecholamine levels and develop heart failure by 6 months of age. [Reprinted with permission from *Nature* (36)]

subtype and cell type. The acute effects of  $\alpha_1$ AR stimulation on the contractile properties of the heart are complex and dependent on the species being studied (18). Activation of cardiac  $\alpha_1$ ARs protects myocytes against ischemic and other injuries through activation of PKC (19). All three  $\alpha_1$ AR subtypes have been disrupted in mice (20). Cardiac hypertrophy normally observed with chronic administration of norepinephrine is absent in  $\alpha_{1B}$ AR KO mice (21). Moreover, mice lacking both  $\alpha_{1A}$  and  $\alpha_{1B}$ AR have been reported to have abnormal postnatal cardiac development (22).

Chronic activation of  $\alpha_1$ AR and  $\beta$ AR stimulates cardiac myocyte hypertrophy in animals. Neonatal rat ventricular myocytes have been the model system of choice to study signaling pathways leading from  $\alpha_1$ AR

and  $\beta$ AR stimulation to hypertrophy (3, 23). The proposed signaling pathways leading from  $\alpha_1$ AR activation to hypertrophy are complex and may involve activation of extracellular signal-regulated protein kinase (ERK) and PI3K through  $G_q$ , PKC, and Ras, and activation of calcineurin through  $Ca^{2+}$  and calmodulin. Both pathways lead to changes in gene expression through activation of transcription factors (4, 5). RhoA and Rac1 may also be involved in myocyte hypertrophy by affecting gene expression, the cytoskeleton, or both (5). The mechanism of  $\beta$ AR contribution to myocyte hypertrophy is less well understood. Activation of  $G_s$  by  $\beta_1$  and  $\beta_2$ ARs and  $G_i$  by  $\beta_2$ ARs may both be involved (3).

There is a growing body of evidence that subtype-specific signaling of ARs in cardiac myocytes involves the association of receptors and down-stream signaling molecules in plasma membrane signaling compartments, where signaling molecules are held together by multidomain scaffolding proteins (24). A PDZ domain-binding motif on the C-terminus of the  $\beta_1$ AR selectively binds two families of PDZ domain-containing proteins, including PSD-95 and MAGI-2 (25, 26). A similar interaction is necessary to maintain the integrity of  $\beta_1$ AR to  $G_s$  coupling in myocytes, because disruption of the  $\beta_1$ AR PDZ motif leads to promiscuous coupling to both  $G_s$  and  $G_i$  in neonatal mouse cardiac myocytes (27). On the contrary, data from HEK293 cells suggest that a PDZ motif on the C-terminus of the  $\beta_2$ AR may be involved in recycling internalized  $\beta_2$ ARs and for the receptor-mediated activation of  $Na^+$ - $H^+$  exchanger (28, 29). In vitro studies show that the  $\beta_2$ AR also associates with the scaffold protein AKAP (A kinase anchoring protein), which may link  $\beta_2$ ARs with other signaling and regulatory proteins in cardiac myocytes (30, 31). Moreover,  $\beta_2$ AR associates with caveolin-3 in neonatal mouse cardiac myocytes, and disruption of caveolar structures enhances the coupling  $\beta_2$ AR to  $G_s$  (32–34). Together, these observations support the notion that receptor-associated proteins and the location of receptors in specific plasma membrane microdomains are important for the AR subtype-specific signaling in cardiac myocytes.

The differences in data or interpretation among some studies on AR signaling in cardiac myocytes may in part be due to the many differences between neonatal and adult cardiac myocytes. Thus, characterization of adrenergic signaling in both neonatal and adult cardiac myocytes will be needed to generate a more comprehensive picture of adrenergic signaling pathways in animal hearts. Moreover, differences between receptor homologs and variations in receptor subtype compositions among species may also contribute to the signaling differences observed in different animal model

systems. The Myocyte Adrenergic Signaling Pathway (35) in the STKE Connections Maps should facilitate the effort to understand their physiological function in vivo.

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#### VIEWPOINT

## Regulators of Cerebellar Granule Cell Development Act Through Specific Signaling Pathways

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The proper development of the central nervous system depends upon a finely tuned balance between cell proliferation and programmed cell death (PCD). Although PCD was initially believed to depend solely on the inability of certain neurons to obtain access to a limited supply of trophic factors, it has become apparent that the local production of death signals is also critical. In this Viewpoint, we discuss several pathways implicated in the survival of cerebellar granule cells—both pathways that protect from apoptosis and pathways that promote apoptosis—and describe how these disparate pathways converge on the final common mediators of PCD. Information on other important pathways implicated in granule cell survival may be found in the Connections Maps.

A delicate balance between cell proliferation and PCD is necessary for the harmonious development of the central nervous system (CNS). In both neuronal and glial populations, as many as 70% of the generated cells die during the developmental period (1). In the adult, dysregulated PCD is thought to be responsible for various neurological diseases including epilepsy, Alzheimer's disease, and Parkinson's disease (2). It was initially proposed that neuronal apoptosis resulted from growth factor deprivation, and it was generally assumed that only neurons that establish correct synaptic connections were able to obtain the trophic support required for survival. But there is increasing evidence that local production of death signals is essential for the removal of surplus neurons that occurs

during morphogenesis of the nervous system (3). Characterizing the signaling pathways involved in the actions of neurotrophic factors and death molecules is therefore critical for understanding the mechanisms underlying normal brain development and neurodegenerative diseases.

The cerebellar cortex of newborn rat, one of the best-studied regions of the developing brain (4), is a model well suited to identifying factors that control neuronal differentiation and apoptosis. In particular, cerebellar granule cells, which constitute the most abundant neuronal population in the mammalian CNS, have the advantages of a cell line without the drawbacks of transformed tumor cells. Granule cells have thus been widely used to investigate the neurotrophic or neurotoxic effects of various factors and have provided crucial information regarding the basic molecular interplay of the cell death machinery. This Viewpoint is not meant to be comprehensive, but rather to cover a few of the most important granule cell survival pathways.

#### Neurotrophic Factors

Many factors that inhibit cerebellar granule cell apoptosis have now been identified; these include growth factors, neurotransmitters, and neuropeptides (Fig. 1, top left). One of the first granule cell neuroprotectors characterized was insulin-like growth factor-1 (IGF-1) (5). IGF-1 is synthesized and released by Purkinje cells, which settle in the cerebellum earlier than granule cells. It is assumed that, in vivo, IGF-1 secreted by Purkinje cells exerts a neurotrophic effect on cerebellar granule cells during their migration from the proliferative zone (the external granule cell layer) to their final destination (the internal granule cell layer). Several neurotrophins prevent cerebellar granule cell death in a temporally distinct manner. For example, brain-derived neurotrophic factor (BDNF) and neurotrophins 4 and 5 (NT-4 and NT-5) exert neuroprotective activities on immature neurons, whereas only NT-3 prevents the death of differentiated neurons (6). Some neuromediators have more ambiguous effects. For instance, release of the excitatory neurotransmitter glutamate at newly established synapses is critical for cell survival after initial assembly of the neuronal network, whereas, in differentiated granule cells, concentrations of glutamate that were neuroprotective earlier in development provoke a sustained and toxic increase of intracellular calcium (7).

The two predominant signaling pathways activated by growth factors in cerebellar granule cells are the phosphoinositide 3-kinase

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