Interplay between electrical activity and bone morphogenetic protein signaling regulates spinal neuron differentiation

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A gradient of bone morphogenetic proteins (BMPs) along the dorsoventral axis of the spinal cord is necessary for the specification of dorsal neurons. Concurrently, a gradient of calcium-mediated electrical activity is present in the developing spinal cord but in an opposing ventrodorsal direction. Whether BMPs and electrical activity interact in embryonic spinal neurons remains unknown. We show that BMP decreases electrical activity by enhancing p38 MAPK-mediated negative modulation of voltage-gated sodium channels. In turn, electrical activity affects the phosphorylation status and nuclear level of activated Smads, the canonical components of BMP signaling. This interaction between calcium spike activity and BMP signaling regulates the specification of the dorsal commissural spinal neuron phenotype. The present study identifies an unexpected interplay between BMPs and electrical activity that is critical for decoding the morphogen gradient during spinal neuron differentiation.

calcium signaling | spinal interneuron | mitogen-activated protein kinase | noggin | activity-dependent neuronal specification

Nervous system function relies on connections among specialized cells that acquire their distinctive identity during embryonic development. Specialization of neurons originates with the specification of neural progenitors driven by secreted factors present as gradients along the main axes of the developing nervous system. Prototypical of these molecules are bone morphogenetic proteins (BMPs), members of the TGF-β superfamily (1). Among the numerous BMPs, *Bmp2*, *Bmp4*, *Bmp7*, growth differentiation factor 7 (*Gdf7*), activin, and dorsalin are expressed in the roof plate during neural tube development (1–3). *In vitro* and *in vivo* experiments in various organisms have shown that the dorsoventral BMP gradient (4, 5) is essential for specification of dorsal sensory neurons and interneurons, such as the commissural interneurons (1, 3, 6). The mechanisms by which BMP gradient is deciphered remain unclear.

BMP ligands form a homomeric or heteromeric complex that binds to two types of transmembrane serine-threonine kinase receptor, the type I (BMPRIA and BMPRIB) and type II (BMPRII) receptors (7). Following BMP binding, the type II receptor phosphorylates the type I receptor (8). This process leads to the C-terminal phosphorylation of the pathway-restricted Smads (R-Smads, Smads1, -5, or -8), which are then released from the receptor and recruit the common mediator Smad (Co-Smad, Smad4) into a complex. This complex migrates into the nucleus and activates the transcription of specific target genes (9, 10), constituting the canonical BMP signaling pathway. BMP can also recruit the MAPK pathway through receptor-mediated phosphorylation of Tak1, leading to the activation of p38 MAPK (11, 12). BMP stimulation has also been shown to activate the Erk1/2 and LIM kinases in certain cell types (13, 14). The role of BMP noncanonical pathways during dorsoventral spinal phenotype differentiation and whether different signaling cascades function in concert or independently from each other has not been determined.

Concurrently, while neurons are being generated and specialized, they exhibit spontaneous Ca^{2+}-mediated electrical activity (15). This activity occurs prior and during synapse formation and modulates several aspects of nervous system development. It is important for the proliferation of mouse cortical progenitors (16), cell migration of cerebellar, hippocampal, and cortical cells in mice (17–19), differentiation of spinal and cerebral neurons in *Xenopus* and mouse (20–25), and pathfinding of retinogeniculate projections in ferret and of motor neuron axons in chicken (26, 27), demonstrating the universality of the relevance of early electrical activity for nervous system development. Although the role of electrical activity in neuronal development is now generally accepted, it is still considered to be important mostly in later stages of circuit formation; the early neuronal specialization is believed to occur solely based on morphogenetic protein signaling and independently of electrical activity.

In the *Xenopus* embryonic spinal cord, Ca^{2+} spike activity consisting of rapid transients in intracellular [Ca^{2+}] that are propagated throughout the whole neuronal cell body, lasting for 20 s, becomes apparent after neural tube closure (21) and is present in a ventrodorsal gradient (20, 21) opposite to that of BMPs (4). In the present study, we show that there is an interplay between Ca^{2+}-mediated electrical activity and BMP signaling that is important for the appropriate differentiation of spinal neurons.

Results

BMP Signaling Acutely Modulates Ca^{2+} Spike Activity in Embryonic Spinal Neurons. To determine whether BMPs contribute to the gradient of excitability along the dorsoventral axis of the embryonic spinal cord (20, 21), we imaged Ca^{2+} dynamics on the dorsal and ventral surfaces of the developing *Xenopus* spinal cord following exposure to the exogenous BMP4/7 heterodimer. BMP4/7 acutely decreases Ca^{2+} spike activity of spinal neurons [the only spinal cell type that exhibits Ca^{2+} spikes during these developmental stages (20)] and this is blocked by Noggin, a BMP antagonist, which in the absence of exogenous BMP increases Ca^{2+} spike activity of ventral (Fig. 1 *A* and *G*) and dorsal (see Fig. 3) neurons. The effect of BMP is dose-dependent and evident in both dorsal and ventral spinal cells (Fig. 1*B*). Expression of a constitutively active form of the BMP receptor, Alk3 (28), during the period of spontaneous Ca^{2+} spiking (Fig. S1) mimics BMP-induced decrease in electrical activity. This finding is

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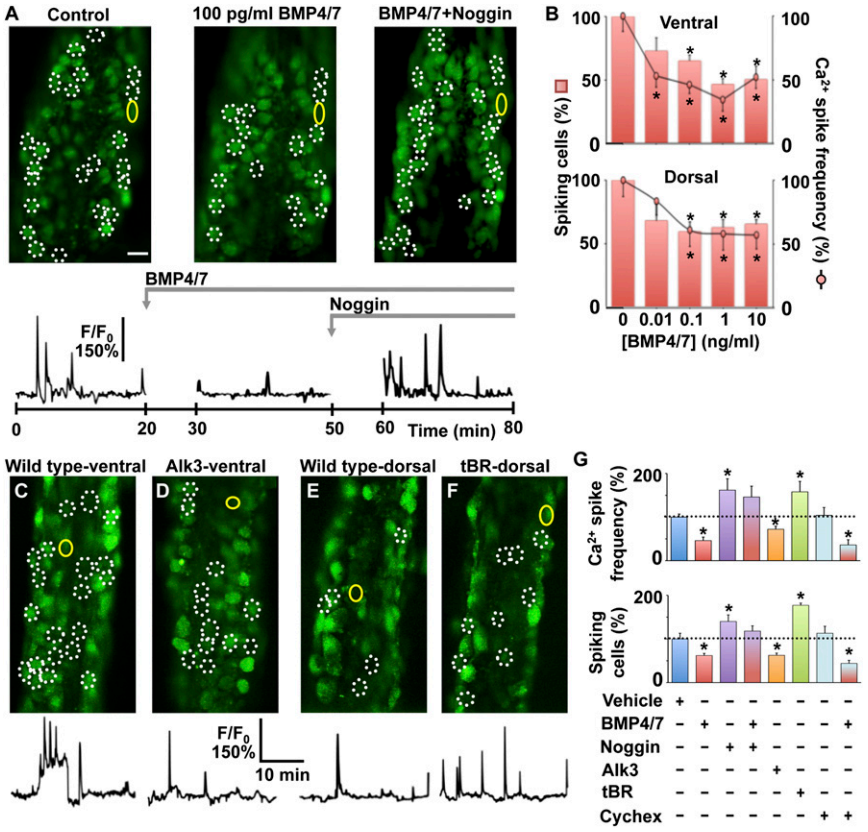

Fig. 1. BMP signaling decreases Ca^{2+} spike activity in the developing spinal cord. Neural tubes from stage-24 (26 h postfertilization) embryos were loaded with Fluo4-AM and confocally imaged. (*A*) Ventral view of a single neural tube imaged consecutively for 20-min intervals under control conditions, in the presence of 100 pg/mL BMP4/7 and a combination of BMP4/7 and 500 ng/mL Noggin. Traces show Ca^{2+} spike activity for a single cell outlined in yellow. In *A* and *C–F*, circles identify cells spiking during recording. (Scale bar, 20 μm.) (*B*) Dose-dependent effect of BMP4/7 on Ca^{2+} spike activity. Ca^{2+} spike incidence and frequency in ventral and dorsal neural tube are 12 ± 2 vs. 6 ± 0.7 spiking cells during 30-min recordings and 7 ± 0.8 vs. 3 ± 0.4 Ca^{2+} spikes per cell per hour, respectively (20, 21). (*C–F*) Representative examples of Ca^{2+}-imaged neural tubes from wild-type (*C* and *E*) and inducible BMPRIA mutants, Alk3 (*D*), and tBR (*F*). Traces show Ca^{2+} spike activity for cells outlined in yellow. (*G*) Enhancing or inhibiting BMP signaling exerts opposite effects on Ca^{2+} spike activity and is translation-independent. Cychex, cycloheximide; BMP4/7, 10 ng/mL; Noggin, 500 ng/mL; Cychex, 10 μg/mL. Results are comparable in dorsal and ventral samples. Data in *B* and *G* are mean ± SEM percent of spiking cells and spike frequency compared with control (30-min recording before addition of agents or recording from sibling wild-type embryos, dashed line in *G*), $n \geq 5$ embryos per experimental group, $^*P < 0.01$.

evident even in ventral neurons (Fig. 1 *C*, *D*, and *G*), which in wild-type embryos exhibit higher levels of electrical activity than their dorsal counterparts (20, 21). In contrast, expression of a dominant-negative form of BMP receptor, tBR (28) (Fig. S1), increases Ca^{2+} spike activity even in dorsal neurons (Fig. 1 *E–G*), which exhibit lower levels of electrical activity in wild-type embryos compared with ventral cells (20, 21). The BMP-induced decrease in Ca^{2+} spike activity occurs within minutes of BMP4/7 application, and cycloheximide, a translation inhibitor, fails to block this decrease (Fig. 1*G*). These results indicate that BMPs acutely decrease Ca^{2+} spike activity through a translation-independent mechanism in embryonic spinal neurons, and suggest that the endogenous dorsoventral BMP gradient contributes to the gradient of Ca^{2+} spike activity along the ventrodorsal axis of the developing spinal cord.

Role of MAPKs and Voltage-Gated Ion Channels in the BMP-Induced Decrease in Ca^{2+} Spike Activity. Intracellular transduction of BMP signal is mediated by the canonical Smad pathway or by the noncanonical MAPK-driven cascade (9–12). Although Smads have been almost universally found to mediate BMP action, the participation of MAPKs depends on tissue or cell type and in some cases remains undetermined. To elucidate the molecular mechanisms underlying BMP-induced decrease in Ca^{2+} spike activity, we examined the role of MAPKs and Smad1/5/8 on the effect of BMP4/7. Inhibition of p38 MAPK by two different inhibitors prevents the BMP-induced decrease in Ca^{2+} spike activity, while pharmacological inhibition of the MEK1/Erk1/2 pathway decreases electrical activity and occludes the effect of BMP (Fig. 2*A*). On the other hand, inhibition of JNK or Smad1/5/8 phosphorylation (29) does not prevent BMP action (Fig. 2*A*). The profile of changes in the number of spiking cells is similar to the changes observed in Ca^{2+} spike frequency upon treatments (Fig. 2*A*) and is evident in the dorsal (Fig. 2*A*) and ventral (Fig. S2) spinal cord. Notably, all of the MAPK inhibitors used affect basal levels of Ca^{2+} spikes, in contrast to the Smad1/5/8 inhibitor, which does not exert any effect, suggesting that the endogenous activity of MAPKs regulates spontaneous Ca^{2+} spike activity. In addition, BMP4/7 increases the level of P-p38 and decreases the level of P-Erk1/2 in developing neural tubes (Fig. 2*B*). These data support the involvement of either activation of p38 or inhibition of Erk1/2 as mediators of BMP-induced decrease in Ca^{2+} spikes. To unravel which of these two MAPKs mediate the BMP effect, we expressed a dominant-negative form of p38 [DN-p38, Flag p38-α (agf) (30)] or a constitutively active (CA) form of MEK1 [CA-MEK, Mek1 R4F fused to estrogen receptor (ER) (31)], which renders higher levels of activated Erk1/2, during the period of spontaneous electrical activity and assessed the effect of BMP on the background of these mutant MAPK forms (Fig. S3). Expression of DN-p38 or CA-MEK increases spontaneous Ca^{2+} spike activity (Fig. 2*C*), in agreement with the effect of pharmacological inhibitors of p38 and MEK1 (Fig. 2*A*). The effect of BMP on electrical activity is abolished in neural tubes in which p38 activity is down-regulated, but in embryos, in which Erk1/2 is constitutively active BMP still decreases Ca^{2+} spike activity (Fig. 2*C* and Fig. S3).

p38 and Erk1/2 phosphorylate voltage-gated Na^+ and Ca^{2+} channels (Na_v and Ca_v), respectively (32, 33). Both Na_v and Ca_v are important for the manifestation of Ca^{2+} spikes (34). p38 modulates $Na_v1.6$ activity by phosphorylating its α-subunit and decreasing its current density (33). We find that overexpression of wild-type $Na_v1.6$ (Fig. S4) increases spontaneous Ca^{2+} spike activity and addition of BMP4/7 prevents this increase (Fig. 2*D*). On the other hand, mutating the p38 phosphorylation site in $Na_v1.6$ (Fig. S4) abolishes BMP action on spikes (Fig. 2*D*). These results suggest that BMP may regulate electrical activity by modulating Na_v activity. However, overexpression approaches generally override endogenous mechanisms. Therefore, we cannot conclude whether $Na_v1.6$ is the endogenous target channel for

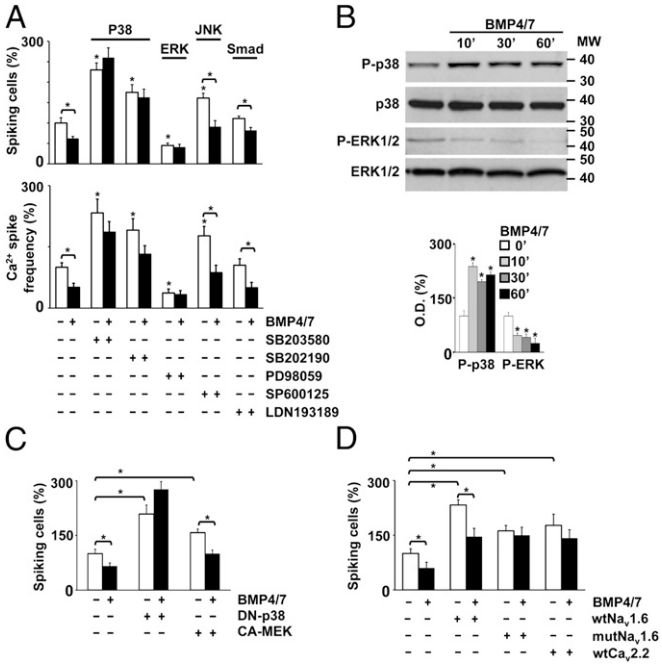

Fig. 2. The negative modulation of voltage-gated Na^+ channel by p38 MAPK participates in the BMP-induced decrease in Ca^{2+} spike activity. (*A*) BMP action on Ca^{2+} spike activity in the presence of kinase inhibitors. Data are mean ± SEM percent of spiking cells and percent of Ca^{2+} spike frequency in dorsal spinal cord compared with control (30 min prior addition of drug), $*P < 0.001$. Similar profile of changes in Ca^{2+} spike activity upon treatments is observed in the ventral spinal cord (Fig. S2). (*B*) BMP4/7 affects phosphorylation status of p38 and Erk1/2 in neural tube explants from stage-24 embryos, as shown in representative Western blots. Graph shows mean ± SEM percent OD, previously normalized to the level of total p38 or Erk1/2, compared with control (0′ BMP4/7), $*P < 0.0001$. (*C*) Ca^{2+} spike activity in dorsal neural tubes from wild-type control, dominant-negative (DN) p38, and CA-MEK–expressing embryos (stage 24). Data are mean ± SEM percent of spiking cells compared with wild-type control, $*P < 0.05$. (*D*) Overexpression of mutant Nav1.6 at the p38 phosphorylation site prevents BMP-induced decrease in Ca^{2+} spikes. Ca^{2+} spike activity in neural tubes from wild-type (wt) control, wtNa_v1.6-, mutated (mut)Na_v1.6-, and wtCa_v2.2-overexpressing embryos (stage 24). Data are mean ± SEM percent of spiking cells compared with wt control, $*P < 0.01$. $n = 5$ per experimental group, BMP4/7: 10 ng/mL.

BMP-induced decrease in Ca^{2+} spike activity. Overexpression of wild-type Ca_v2.2 (Fig. S4) increases spontaneous Ca^{2+} spike activity and BMP is unable to decrease it (Fig. 2*D*). Ca_v activation occurs downstream of Na_v opening during Ca^{2+} spike generation (34). Hence, the inability of BMP to decrease Ca^{2+} spike activity when Ca_v is overexpressed supports the potential model of BMP acting upstream of Ca_v.

Taken together, these results point toward p38-mediated negative modulation of Na_v as a possible mechanism through which BMP decreases Ca^{2+} spikes.

Suppression of Electrical Activity Expands Differentiation of the Dorsal Commissural Neuron Population to Ventral Domains, Paralleling the Effect of Ectopic BMP. BMP signaling in dorsal spinal cells promotes expression of Lh2A/B, a LIM homeodomain transcription factor expressed in dorsal commissural neurons (35, 36). We analyzed the effect of perturbing electrical activity on the differentiation of Lh2A/B-expressing neurons. Suppression of Ca^{2+} spike activity with voltage-gated Na^+ and Ca^{2+} channel blockers (VGCblock, listed in *SI Materials and Methods*) increases the number of cells expressing Lh2A/B and extends this phenotype to more ventral domains (Fig. 3). These changes in Lh2A/B expression resemble those induced by ectopic BMP (Fig. 3). The expansion of Lh2A/B expression to ventral domains cannot be explained by a sole perturbation in cell migration because there is a net increase in the number of Lh2A/B-expressing cells rather than a mere change in their distribution.

Enhancement of electrical activity with veratridine (a Na^+ channel agonist that depolarizes neurons and promotes activation of Ca_v, triggering Ca^{2+} spikes), or inhibition of BMP signaling with the BMP antagonist, Noggin, or an inhibitor of BMP-mediated Smad signaling, does not change significantly the number of Lh2A/B-immunopositive cells (Fig. 3). Perturbations of BMP signaling and Ca^{2+} spike activity were implemented following neural tube closure, when Ca^{2+} spikes become apparent, to avoid interfering with BMP action on earlier embryonic stages. Thus, this lack of effect may be because of the inability to down-regulate Lh2A/B expression in cells that were already specified before treatments, or this basal expression of Lh2A/B may be independent of BMP signaling and electrical activity.

None of the implemented perturbations in activity or BMP signaling affect the number of neural progenitors (Sox2-expressing cells) or total number of spinal cells (DAPI-labeled nuclei) (Fig. S5), suggesting that the increase in number of Lh2A/B-expressing cells when activity is suppressed and BMP signaling is enhanced is not caused by changes in cell proliferation. This finding concurs with the fact that Ca^{2+} spike activity is not apparent in progenitors and that changes in electrical activity do not affect number of ventral progenitors such as Nkx2.2- and Nkx6.1-expressing cells (20).

Increase in the Number of Lh2A/B-Expressing Neurons Requires Both BMP Canonical Signaling and Suppression of Electrical Activity. We next investigated whether the effects of suppressing electrical activity and enhancing BMP signaling on the regulation of Lh2A/B expression are sequential or belong to independent pathways (Fig. S6). Our results show that the expansion of Lh2A/B expression induced by ectopic BMP is blocked by compensating for the BMP-mediated decrease in Ca^{2+} spikes, using a voltage-gated Na^+ channel agonist, veratridine (Fig. 3). This finding suggests that BMP- and electrical activity-dependent regulation of Lh2A/B expression are not independent pathways (Fig. S6*A*). However, this result still enables two scenarios: one in which suppression of Ca^{2+} spikes is linearly downstream of BMP signaling and hence sufficient to induce Lh2A/B expression (Fig. S6*B*), or another one in which decrease in Ca^{2+} spikes, although still downstream of BMP trigger, synergizes with BMP to induce the dorsal phenotype expression (Fig. S6*C*). To sort through these possibilities, we studied the effect of combining suppression of electrical activity with inhibition of BMP canonical signaling pathway. We find that expansion of Lh2A/B expression induced by suppressing electrical activity is prevented by antagonizing the BMP canonical pathway, either with Noggin (which sequesters endogenous BMP) or with a Smad1/5/8-specific inhibitor (Fig. 3), thus favoring a mechanism of synergistic interaction between BMP signaling and suppression of Ca^{2+} spikes for driving the dorsal phenotype (Fig. S6*C*). These findings indicate that the interplay between electrical activity and BMP signaling regulates dorsal commissural neuron specification. Furthermore, both BMP and suppression of Ca^{2+} spike activity are necessary, and neither of them are singly sufficient to induce ectopic expression of Lh2A/B in the developing spinal cord. These data suggest that high levels of electrical activity are crucial to prevent the commissural dorsal phenotype expression in ventral neuron domains and that, in the absence of this activity, the otherwise relatively low levels of BMP present ventrally are enough to recruit ventral cells to this dorsal phenotype.

The canonical BMP signaling pathway is driven through receptor-mediated phosphorylation of Smad1/5/8 in the carboxyl-terminal domain (P-tail-Smad) and its subsequent translocation to the nucleus for the regulation of target gene expression (10). Translocation of active Smads to the nucleus is prevented by

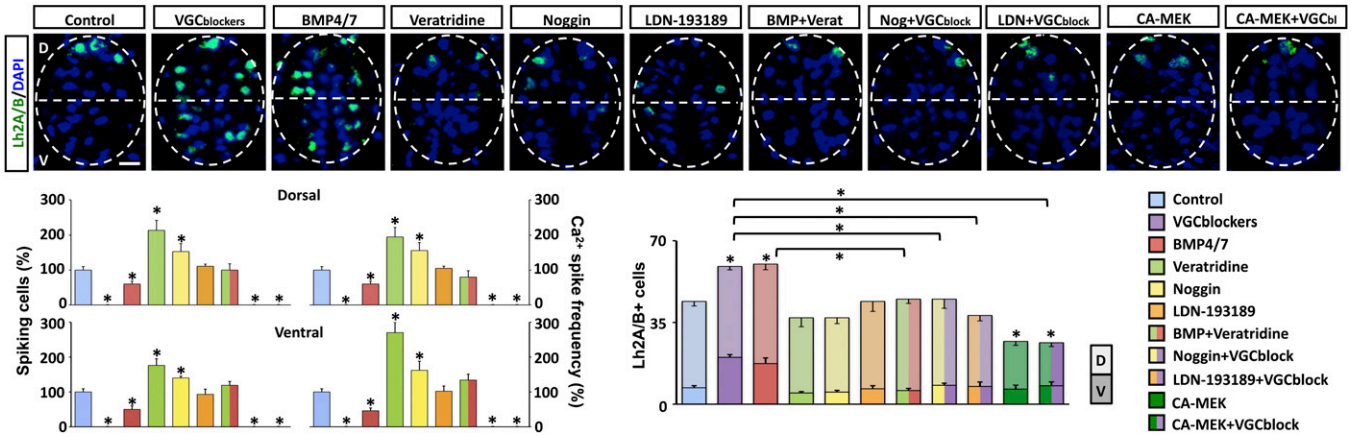

Fig. 3. The interplay between Ca^{2+} spike activity and BMP signaling regulates differentiation of Lh2A/B-expressing cells. Immunostaining of transverse sections of the spinal cord (outlined) from wild-type embryos or CA-MEK–expressing embryos treated with the indicated agents: VGCblock, voltage-gated Na^{+} and Ca^{2+} channel blockers; Verat, veratridine; BMP+verat, BMP4/7 and veratridine; Nog+VGCblock, Noggin and VGCblock; LDN+VGCblock, LDN-193189 and VGCblock. Graphs show mean ± SEM percent of spiking cells and Ca^{2+} spike frequency in the dorsal and ventral spinal cord compared with control (30-min recording before addition of agents), $n \geq 5$ stage-24 (26-h-old) embryos and mean ± SEM of Lh2A/B immunopositive cells/100 μm of dorsal and ventral spinal cord, $n \geq 5$ stage-35 (48-h-old) larva per experimental group, $^{*}P < 0.05$. D, dorsal; V, ventral. (Scale bar, 20 μm.) Concentrations of agents used to impregnate beads are indicated in *SI Materials and Methods*. For Ca^{2+} imaging experiments: VGCblock, indicated in *SI Materials and Methods*; BMP4/7, 10 ng/mL; Veratridine, 1 μM; Noggin, 1 and 0.5 μg/mL, dorsal and ventral, respectively; LDN-193189, 5 μM.

Erk1/2-mediated phosphorylation of the Smad linker site (37), and we find that expression of CA-MEK, which renders a constitutively phosphorylated Erk1/2 (Fig. S3 *C*, and *D*), decreases the level of BMP-induced P-tail-Smad in developing neural tubes (Fig. S7). Levels of electrical activity modulate levels of active Erk1/2, which is higher in ventrolateral domains of the developing spinal cord (Fig. S8), where Ca^{2+} spike activity is higher. Interestingly, expression of CA-MEK prevents the effect of suppressors of Ca^{2+} spikes on Lh2A/B specification (Fig. 3). These results suggest that the level of Erk1/2 activation may be relevant to the regulation of Lh2A/B expression by Ca^{2+} spike activity.

Ca^{2+}-Mediated Electrical Activity Modifies the Phosphorylation Status and Subcellular Levels of Smads. In light of this regulation of Lh2A/B expression by Ca^{2+}-mediated electrical activity and the apparent Smad dependence, we investigated the action of Ca^{2+} spike activity on tail-Smad1/5/8 phosphorylation and subcellular localization. We suppressed or enhanced Ca^{2+} spike activity in isolated neural tubes and then processed the tissue for Western blot assays. We find that suppression of Ca^{2+} spike activity enhances the BMP-induced increase in level of P-tail-Smad, while enhancement of activity by preincubating neural tubes with veratridine, followed by coincubation with BMP4/7, decreases it (Fig. 4). These effects are evident only in the nuclear and not in the cytosolic fraction. Suppression of activity induces an increase in nuclear P-tail-Smad levels even in the absence of exogenous BMP (Fig. 4). These results demonstrate that the magnitude of Smad recruitment to the nucleus is not only dependent on BMP level but also on the level of electrical activity.

Discussion

This study demonstrates that BMP signals acutely to embryonic spinal neurons by regulating levels of Ca^{2+} spikes and, thus, contributes to the ventrodorsal gradient of electrical activity. This Ca^{2+} spike-activity gradient regulates the Smad-mediated BMP signaling and is required for limiting BMP-induced expansion of dorsal commissural neuron specification into ventral domains (Fig. 5). The regulation of spinal cell specialization by electrical activity has also been demonstrated in the embryonic chick spinal cord. Inhibition of spontaneous electrical activity down-regulates expression of the transcription factors Lim1 and Islet1 in developing motor neurons (27). Therefore, the accepted model of gradients of morphogenetic proteins driving the program of spinal cell specification needs to be expanded to include gradients of other developmental cues, such as electrical activity along the dorsoventral axis of the spinal cord that then lead to recruitment of specific combination of transcription factors and phenotypic specialization.

Our findings suggest that MAPKs are involved in the interaction between BMP and Ca^{2+}-dependent electrical activity.

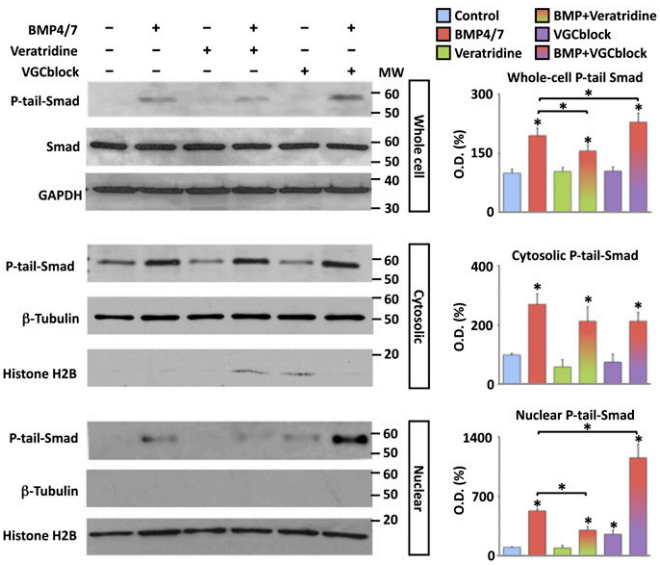

Fig. 4. Ca^{2+} spike activity modulates the phosphorylation status and nuclear levels of activated Smad1/5/8. Western blots of whole-cell, cytosolic, and nuclear extracts from stage-24 neural tube explants incubated with indicated agents for 30 min. Combined treatments consisted of preincubation with veratridine or VGCblock for 30 min before addition of BMP4/7. Graphs show mean ± SEM percent of P-tail-Smad levels normalized to total Smad for whole-cell extracts and to β-tubulin or histone H2B for cytosolic or nuclear fractions, respectively, compared with control, $n > 5$, $^{*}P < 0.001$ compared with control or with BMP-treated samples. VGCblock, indicated in *SI Materials and Methods*; BMP4/7, 10 ng/mL; Veratridine, 1 μM.

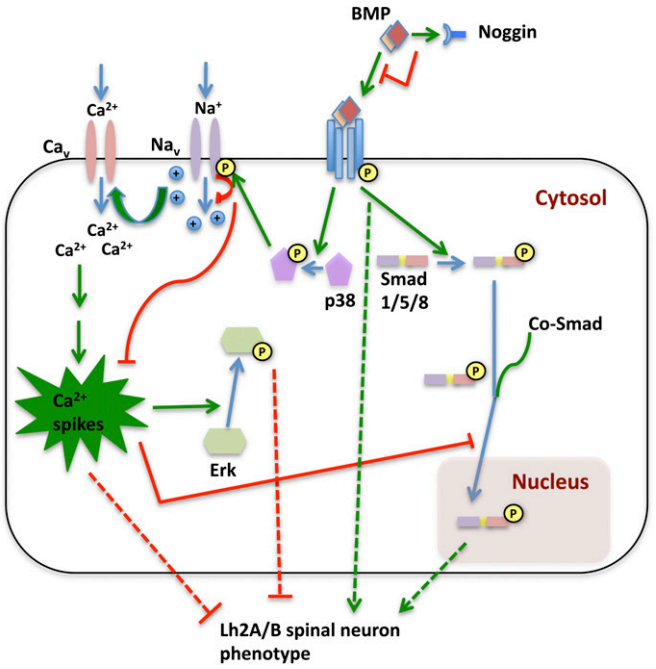

Fig. 5. Proposed model for the possible molecular interplay between BMP and Ca^{2+}-mediated electrical activity pathways during spinal cord development. Binding of BMP to its receptor activates p38 MAPK, which phosphorylates and negatively modulates Na_v activity. This process diminishes the probability of spontaneous Na_v-mediated membrane depolarizing events and, hence, prevents further activation of Ca_v resulting in a decrease in Ca^{2+} spikes. In turn, Ca^{2+} spikes activate Erk1/2 and decrease the level of nuclear P-tail-Smad. The interaction between Ca^{2+} spike activity and BMP signaling regulates the differentiation of the commissural dorsal spinal phenotype.

The points of potential cross-talk could be multiple, from an acute modulation of ion channel activity to a longer-term regulation of gene expression (Fig. 5). Cross-talk between MAPKs and BMPs exists in several systems. BMP4 supports self-renewal of mouse embryonic stem cells by inhibiting the MAPKs Erk1/2 and p38, which, otherwise, negatively modulate Smad1/5-mediated transcription (38). BMP4 and Erk1/2 also interact antagonistically during neural induction in *Xenopus* embryos (39). Other signaling pathways and points of interaction, linking Ca^{2+} signaling and Smad-driven regulation of gene expression have been identified, and may also participate in the interplay presented in this study. Calmodulin interacts with Smad proteins in a Ca^{2+}-dependent manner (40). This interaction results in modulation of Smad1 and Smad2 signaling in developing *Xenopus* embryos that affects early embryonic ventralization and mesodermal differentiation (41, 42). The interaction between electrical activity and Smad signaling may also be implemented at the level of competing for transcriptional coactivators: for example, cAMP responsive element binding protein (CREB), phosphorylation of which is activity-dependent, may squelch coactivators also required by Smad; or by integrating Smad and CREB transcriptional activities at the level of the promoter of a specific gene (43, 44). Further studies are needed to determine the molecular mechanisms used in regulating the differentiation of dorsal commissural neurons and the different levels at which electrical activity and BMP signaling interact.

Specification of dorsoventral spinal cells depends on the integration of Wnts, Sonic hedgehog (Shh), and BMP pathways. Wnts and BMPs antagonizing Shh ventralizing action and, reciprocally, Shh opposing the dorsalizing action of BMPs in the developing spinal cord (45–47). The precise mechanisms underlying their interactions are not fully understood. From this study and the universal character of Ca^{2+} signaling, one may predict that Ca^{2+} dynamics may provide a plausible mechanism for the integration of diverse morphogen signaling pathways. Our previous work shows that in the developing spinal cord Shh exerts an opposite effect on Ca^{2+} spike activity (20) compared with the decrease induced by BMP presented herein. Shh increases Ca^{2+} spike activity by recruiting Gαi and phospholipase C, and by the participation of transient receptor potential channel 1 and Ca^{2+} influx leading to IP3 transients at the primary cilium correlated with Ca^{2+} spikes (20). When considering these two studies, it becomes evident that both morphogenetic proteins contribute in opposing manner to the gradient of Ca^{2+} spike activity, not by a simple cancelling of each other's pathway but by different molecular mechanisms. Having separate mechanistic strategies for modulating Ca^{2+} spike activity may generate a more exquisite fine-tuning of the level of activity. This scenario, in turn, may allow for a more precise read out of the gradients of these concurrent morphogenetic proteins. It will be interesting to determine how Shh and BMP compete for the regulation of Ca^{2+} spike activity and the impact this balancing act has in the differentiation of spinal neurons.

Here we demonstrate that the interaction between BMP and electrical activity regulates the specification of commissural interneurons in the embryonic spinal cord. The advantage of having electrical activity as a regulatory factor of spinal neuron differentiation becomes apparent when considering that the nervous system can readily interpret changes in intrinsic electrical activity or environmental stimuli and adapt accordingly. Indeed, homeostatic changes in neurotransmitter expression in the developing spinal cord and brain occur when electrical activity is modified either by molecular or pharmacological perturbations or by changes in sensory stimuli (21–23). It has been recently shown that differentiation of zebrafish dorsal root ganglion sensory neurons depends on the level of early sensory stimulation through a BDNF-dependent noncell autonomous mechanism (48). Whether the determination of relative numbers of sensory dorsal spinal cells versus ventral neurons directly engaged in motor function is influenced by changes in intrinsic electrical activity and early perception of environmental stimuli remains as an exciting question waiting to be elucidated.

Materials and Methods

Ca^{2+} Imaging. Ca^{2+} imaging was performed as previously described (20, 21). All experiments were approved by the University of California at Davis Institutional Animal Care and Use Committee. The effects of proteins and drugs were assessed by recording for 30 min before and after addition of each agent, with the exception of BMP4/7 treatment followed by BMP4/7+Noggin in which we imaged samples during three intervals (before, after BMP4/7, after BMP4/7+Noggin) of 20 min each. BMP4/7, Noggin (R&D Systems), veratridine, and Na^+ and Ca^{2+} voltage-gated channel blockers (VGC block) were incubated for 10 min and all other drugs were incubated for 45 min.

In Vivo Gene Misexpression. For Alk3 and tBR, 40 pg of γ-crystallin-GFP-hsp70-alk3 or γ-crystallin-GFP-hsp70-tBR plasmids were microinjected in both blastomeres of two-cell–stage embryos. For Dominant-negative p38 (DN-p38-flag), mRNA was synthesized as previously described (20, 21) from Addgene plasmid 20352, PCDNA3 Flag p38-α. For ER-CA-MEK, 100 pg of an inducible CA-MEK (Addgene plasmid 21207: CC2 Mek1 R4F fused to the modified estrogen receptor, ER-CA-MEK), were microinjected in both blastomeres of two-cell–stage embryos. For $Na_v1.6$ and $Ca_v2.2$, mRNA was synthesized as previously described (21). $Na_v1.6$ mutant form was obtained by site-directed mutagenesis with the Quick change XL site directed mutagenesis kit (Stratagene).

Western Blots. Western blots were performed as previously described (20, 49). Fractionation of cytosolic and nuclear protein fractions was done with NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) and samples processed according to manufacturer's protocol.

In Vivo Drug Delivery. In vivo drug delivery was performed as previously described (20, 21, 49). Agarose beads (80 μm; BioRad) were loaded for at least 1 h with the indicated agents. Bead-implanted embryos were grown to stage 35 (2-d-old) when they were fixed and sectioned for immunostaining.

Immunostaining. Samples were processed for immunostaining as previously described (20, 21, 49). Primary antibodies used were: rabbit anti-Lh2A/B (1:3,000; T. M. Jessell laboratory, Columbia University, New York, NY), goat anti-Sox2 (1:100; R&D Systems), and rabbit anti-P-Erk1/2 (1:50; Cell Signaling). Immunoreactive cells were counted in at least 20 consecutive 10-μm sections per embryo.

Data Collection and Statistics. At least five samples were analyzed for each group from at least three independent clutches of embryos. Statistical tests used were paired or unpaired *t* test or ANOVA, when multiple experimental groups were compared simultaneously, $P < 0.05$.

Additional information is available in *SI Materials and Methods*.

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