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Local and long-range endogenous resting potential gradients antagonistically regulate apoptosis and proliferation in the embryonic CNS

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Abstract

Bioelectric signals, particularly transmembrane voltage potentials (V_{mem}), play an important role in large-scale patterning during embryonic development. Endogenous bioelectric gradients across tissues function as instructive factors during eye, brain, and other morphogenetic processes. An important and still poorly-understood aspect is the control of cell behaviors by the voltage states of distant cell groups. Here, experimental alteration of endogenous V_{mem} was induced in *Xenopus laevis* embryos by misexpression of well-characterized ion channel mRNAs, a strategy often used to identify functional roles of V_{mem} gradients during embryonic development and regeneration. Immunofluorescence analysis (for activated caspase 3 and phosphor-histone H3P) on embryonic sections was used to characterize apoptosis and proliferation. Disrupting local bioelectric signals (within the developing neural tube region) increased caspase 3 and decreased H3P in the brain, resulting in brain mispatterning. Disrupting remote (ventral, non-neural region) bioelectric signals decreased caspase 3 and highly increased H3P within the brain, with normal brain patterning. Disrupting both the local and distant bioelectric signals produced antagonistic effects on caspase 3 and H3P. Thus, two components of bioelectric signals regulate apoptosis-proliferation balance within the developing brain and spinal cord: local (developing neural tube region) and distant (ventral non-neural region).Together,the local and long-range bioelectric signals create a binary control system capable of fine-tuning apoptosis and proliferation with the brain and spinal cord to achieve correct pattern and size control. Our data suggest a roadmap for utilizing bioelectric state as a diagnostic modality and convenient intervention parameter for birth defects and degenerative disease states of the CNS.

Keywords

V_{mem}; non-cell-autonomous; apoptosis; proliferation; brain; bioelectricity

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Introduction

A key question in developmental biology concerns the reliable self-assembly of the correct 3-dimentional morphology. The tight coordination of global patterning cues which control cell-level behaviors such as proliferation and apoptosis sculpt organs to acquire the correct size, shape, and orientation relative to each other during embryogenesis. Embryonic central nervous system (CNS) development is an ideal context in which to explore and understand the signals responsible for achieving the necessary target morphology. Developmental mispatterning is the cause of debilitating disorders, such as spina bifida (unclosed neural tube) (Copp and Greene, 2010, Detrait et al., 2005), anencephaly (small brain) (Copp and Greene, 2010, Detrait et al., 2005, Wallingford, 2006) and also increased susceptibility to degenerative disorders like Parkinson's, Alzheimer's, and autism (Doganli et al., 2013, Goldman et al., 2013, Pratt and Khakhalin, 2013). Hence, understanding how large-scale patterning of the brain normally occurs is a pre-requisite for major biomedical breakthroughs addressing birth defects and repairing injuries.

Organ size and morphology, especially in relation to neighboring tissues and the organism as a whole, are precisely controlled in all forms of multicellular life from plants to animals (Stanger, 2008a, Stanger, 2008b). Appropriate organ size is achieved through regulation of proliferation, cell death and remodeling especially during development (Joseph and Hermanson, 2010, Stanger, 2008a, Stanger, 2008b). During CNS development, a precisely orchestrated control of cell proliferation, cell death and cell differentiation results in expansion of the neural progenitor populations ultimately leading to formation of the complex neural structures (brain and spinal cord) from the pseudo-stratified neuroepithelium (Joseph and Hermanson, 2010, Stanger, 2008a, Stanger, 2008b). Gene regulatory networks (Harvey and Hariharan, 2012, Zhao et al., 2011) and physical forces (Stanger, 2008b, Thompson, 1942) have been shown to regulate this complex process. Here we characterize the role of a novel player: transmembrane voltage potential (V_{mem}) patterns across the developing *Xenopus* embryo, in regulating apoptosis and proliferation within the developing brain.

Every cell (not just excitable neurons and muscle cells) has a characteristic (V_{mem}) across their plasma membrane, which is a result of combination of all ion fluxes through channels and pumps. Spatio-temporal gradients of V_{mem} across groups of cells serve as instructive bioelectric signals (Levin, 2013b, Levin, 2014b, Mustard and Levin, 2014). Such patterns of resting potential within living tissues (bioelectric signals) have been studied in the context of their roles in cell migration and wound healing (Cao et al., 2013, Jaffe, 1979, McCaig et al., 2005, Richard B. Borgens, 1989, Zhao et al., 2006) and have long been proposed to direct growth and form *in vivo* (Burr, 1932). Bioelectric signals are implicated in vertebrate appendage regeneration (Adams et al., 2007, Tseng et al., 2010), cancer initiation and metastasis (Binggeli and Weinstein, 1986b, Blackiston et al., 2011, Brackenbury, 2012, Chernet et al., 2014, Chernet and Levin, 2013b, Chernet and Levin, 2014, Lobikin et al., 2012b), left-right patterning (Aw et al., 2008, Aw et al., 2010, Levin et al., 2002), planarian head induction (Beane et al., 2011, Beane et al., 2013, Marsh and Beams, 1947), and eye and brain formation (Nuckels et al., 2009, Pai et al., 2015, Pai et al., 2012a, Pai et al., 2012b). Thus bioelectric signals have been shown to be important regulators of large-scale patterning

and tissue and organ identity (Levin, 2009, Levin, 2013a, Levin and Stevenson, 2012, Tseng and Levin, 2013a).

In the brain, electrical activity within neural precursors shapes neuronal connections within the developing CNS (Borodinsky et al., 2004, Deisseroth et al., 2004, Swapna and Borodinsky, 2012). Previously, bioelectric signals have been shown to be important determinants of nascent brain morphology (Pai et al., 2015 J. Neuro). Here, we analyze the mechanism of this instructive interaction, by characterizing the role of bioelectrical gradient patterns in regulating apoptosis and cell proliferation during early embryonic CNS (brain and spinal cord) development. The *Xenopus laevis* embryo is an excellent model for studying CNS development (Pratt and Khakhalin, 2013) and facilitates cell-level dissection of bioelectrical signals in embryonic developmental events. Our data indicate that bioelectrical signals regulate both apoptosis and proliferation in the developing CNS. Interestingly, both local (within the developing neural tube) bioelectric cell states and distant (ventral) bioelectric cell states are involved in controlling the amount and location of both apoptosis and proliferation within the CNS. Importantly, the local and distant bioelectric signals function counter to each other, forming a binary control system that can fine-tune the extent of apoptosis and proliferation within the CNS to tightly regulate tissue size *in vivo*. These results shed light on a new endogenous developmental mechanism and suggest strategies for modulating growth and form in applications targeting birth defects and degenerative disease states.

Results

Brain development disrupted by local V_{mem} perturbation is reversed by long-distance V_{mem} signaling

A dynamic endogenous bioelectrical prepattern drives craniofacial development in *Xenopus* (Vandenberg et al., 2011). The central neural plate cells exhibit a strong hyperpolarization as the neural plate folds to form the neural tube (Pai et al., 2015, Pai et al., 2012a), and forced deviation from this endogenous V_{mem} pattern causes disruption of endogenous brain development (Pai et al., 2015, Pai et al., 2012a). Given the role of local V_{mem} states (bioelectric signals in the developing brain region) in shaping brain development (Pai et al., 2015, Pai et al., 2012a), and the importance for coordinating brain size with other anatomical features *in vivo*, we asked whether non-local V_{mem} distributions (bioelectric states of cells far away from the developing brain) might affect endogenous brain development across long distances. Experimental alteration of V_{mem} was induced by misexpression of well-characterized ion channel mRNAs, a strategy often used to identify functional roles of V_{mem} during embryonic development and regeneration (Adams and Levin, 2006, Aw et al., 2008, Levin et al., 2002, Pai et al., 2012a, Pai and Levin, 2014, Pai et al., 2012b, Perathoner et al., 2014, Vandenberg et al., 2011). We specifically altered V_{mem} of cells within relevant (local and/or non-local) regions by injecting mRNAs encoding the hyperpolarizing channel *Kv1.5* [voltage-gated potassium channel (Strutz-Seebohm et al., 2007)]. The effect of introducing this ion channel mRNA on V_{mem} of cranial and other cells in the embryo has previously been characterized at stages 10-21 using the voltage reporter dyes and electrophysiology (Pai et al., 2015, Pai et al., 2012a), confirming its ability to efficiently hyperpolarize

expressing cells. In vivo, *Kv1.5* misexpression eliminates the endogenous V_{mem} differences and spatial gradients within the group of channel expressing cells, driving expressing cells to ~−58 mV, from an endogenous polarization level of ~−20 mV or ~−50 mV of cells outside and inside the prospective brain respectively.

Kv1.5 mRNA (titrated to the lowest levels that produced brain phenotypes) was injected into the dorsal two cells of four-celled embryos [the blastomeres from which neural tissue is derived - local (Moody, 1987)], or the ventral two cells of four-celled embryos [blastomere which do not contribute to neural tissue – non-local; (Moody, 1987)], or both dorsal and ventral blastomeres (Fig. 1A). As previously documented by us and others, we saw no sign of general toxicity or non-specific ill health [midline patterning, scale, proportions and overall growth were normal; (Adams and Levin, 2013, Blackiston et al., 2011, Pai et al., 2012a)(Pai et al., 2015, Pai et al., 2012a)]. Uninjected and water-injected embryos served as controls. To document even subtle changes in brain tissue morphology, we used a transgenic frog line PNTub-GFP (Lin et al., 2012, Marsh-Armstrong et al., 1999), where the neural tubulin promoter drives GFP expression giving rise to tadpoles with GFP-labeled CNS tissue (Fig. 1B). This allowed clear visualization of all *Xenopus* brain and spinal structures, and any deviation from normal patterning.

To determine whether ventral (non-neural) regions' V_{mem} patterns could affect dorsal neural tissue development, *Kv1.5*-injected (dorsal, ventral, or both) embryos were allowed to develop to stage 45, and brain morphology was evaluated (Fig. 1). Control tadpoles had intact anterior neural tissue with well-formed nostrils, olfactory bulbs/forebrain, midbrain, hindbrain and spinal cord (Fig. 1Bi; (Pratt and Khakhalin, 2013). As expected (Pai et al., 2015, Pai et al., 2012a), introduction of ~3-4 ng *Kv1.5* mRNA in the dorsal blastomeres caused high incidence of disrupted endogenous brain tissue formation (~60%; Fig. 1A). The phenotypes included: absence of nostrils, olfactory bulbs, and forebrain, with severely malformed midbrain (Fig. 1Bii-vii). Eye development was also affected, resulting in incompletely formed eyes, eyes fused to the brain and pigmented optic nerves (Fig. 1Bii-vii). However, because eye development is dependent on proper neural development (Fuhrmann, Harada et al., 2007, Zuber, 2010), it is likely that the eye defects may be a secondary manifestation of brain defects. Ventral *Kv1.5* injections alone *reduced* (albeit non-significantly) the background incidence of brain phenotype in un-manipulated controls (Fig. 1A).

Interestingly, ventral *Kv1.5* mRNA injections resulted in near complete prevention (~10%; Fig. 1A) of brain phenotypes induced by concomitant dorsal *Kv1.5* injections. These embryos, which were injected in both - dorsal and ventral blastomeres - show normal brain morphology similar to that of controls, with distinct nostrils, well-formed olfactory bulbs/ forebrain, midbrain and hindbrain (Fig. 1Bviii). These data show that brain mispatterning caused by hyperpolarizing local (dorsal) region can be rescued/prevented by hyperpolarizing the distant (ventral) cells. These data confirm the importance of resting potentials for brain development, and demonstrates that the relevant V_{mem} is not merely that of the cells that actually form the brain: the bioelectric state of cells on the opposite side of the body matters as well, and indeed is able to completely rescue defects in local bioelectric state. The rescue

rules out simple toxicity of channel misexpression as the explanation for the brain defect phenotype, and reveals a long-range signaling component to brain development.

Both local and distant V_{mem} signals regulate apoptosis in the developing brain

What common cellular level processes could be regulated by the local and distant V_{mem} patterns to control embryonic brain development? The extent and pattern of apoptosis is a major factor that regulates tissue sculpting and tissue boundaries in developmental organ formation (Arya and White, 2015, Cowan and Roskams, 2004, Joseph and Hermanson, 2010, Miura, 2011, Nonomura et al., 2013, Perez-Garijo et al., 2013). Importantly, apoptosis is now known to be required for specific morphogenetic events such as regeneration (Bergmann and Steller, 2010, Chera et al., 2009, Tseng et al., 2007) – it is a constructive process, not merely a sign of ill health. Hence, we analyzed apoptosis within the developing brain of V_{mem}-perturbed (hyperpolarized by *Kv1.5* mRNA injection) *Xenopus* embryos, by immunostaining transverse sections through developing brain with an activated caspase 3 apoptosis marker (Porter and Janicke, 1999). Sections anterior/through the eye were used for analysis of developing brain tissue. In control (uninjected) embryos, active caspase 3 was found intermittently and sparsely distributed throughout the developing brain tissue (Fig. 2Ai). This pattern of apoptosis in the developing brain is consistent with previous reports of a low background of apoptosis involved in proper brain development (Chan et al., 2002, Nonomura et al., 2013, Rakic and Zecevic, 2000). Embryos injected with *Kv1.5* (hyperpolarizing) mRNA into the dorsal two cells at four-cell stage (for targeting neural tissues) showed a highly significant increase in active caspase 3 signal within the embryonic brain (Fig. 2Aiii,B; $n>11$ embryos for each experimental group, one-way ANOVA, $p<0.001$, with post-tests). In addition to brain, elevated active caspase 3 signal was also seen in the regions immediately surrounding the brain region (Fig. 2Aiii). We verified our targeting of dorsal tissue by co-injection of β-*galactosidase* mRNA along with *Kv1.5* and then performing the active caspase 3 immunostaining on sections of β-galactosidase-stained embryos (Fig. 2Ci-ii).

To get a better understanding of which specific tissues around the brain were exhibiting upregulation of apoptosis, we obtained thin sections through the brain after the embryos were embedded in paraffin and immunostained for active caspase 3 (Fig. 2D). In comparison to uninjected controls (Fig. 2Di) the active caspase 3 staining in dorsally *Kv1.5*-injected embryos was increased in the brain (as expected), but also in the region around the notochord, and in the developing eye (Fig. 2Dii). Injecting *Kv1.5* (hyperpolarizing) mRNA in only one (left) dorsal cell of the embryo increases active caspase 3 signal only on the left brain of the embryo with the contralateral right side of the brain unchanged (Fig. 2Aii and Ciii), suggesting a local mode of action of dorsal V_{mem} signal in inducing apoptosis within the brain. Surprisingly, *Kv1.5* (hyperpolarizing) mRNA injections in the ventral two blastomeres resulted in a significant *decrease* in the active caspase 3 signal in the developing brain in comparison to the controls (Fig. 2Aiv and B). Remarkably, injecting *Kv1.5* (hyperpolarizing) mRNA in the both dorsal and ventral blastomeres showed significantly decreased active caspase 3 signal in comparison to the dorsal blastomeres injected embryos (Fig. 2A, B and D).

These results suggest that the dorsal V_{mem} pattern acts locally in regulating the extent and pattern of apoptosis in developing brain tissue. The ventral V_{mem} pattern also regulates apoptosis in the developing brain tissue over long distance. Crucially, the dorsal and ventral V_{mem} patterns act counter to each other, in regulating the caspase 3 signal within the developing brain: remote hyperpolarization is sufficient to rescue local induction of apoptosis.

Morphological brain defects induced by V_{mem} perturbation are largely due to V_{mem} regulation of apoptosis

We next asked: to what extent are the brain morphological defects observed upon perturbing the dorsal V_{mem} patterns explained by the voltage-dependent apoptosis? We injected *Kv1.5* (hyperpolarizing) mRNA into two dorsal cells of four-cell embryos to disrupt normal brain development as before [Fig. 1 (Pai et al., 2015, Pai et al., 2012a)]. The chemical apoptosis inhibitor [M50054; (Tsuda et al., 2001)] was then used, from stage 10-30 (corresponding to neural tissue development), to block apoptosis in a suppression analysis strategy. The concentration of the apoptosis inhibitor used was that which did not produce any brain morphological defects by itself (Fig. 3A). The embryos were allowed to develop until stage 45 and scored for brain morphology defects as previously documented (Fig. 1). Dorsal blastomere injections of ~2.2-3 ng *Kv1.5* mRNA resulted in significant increase in the incidence of misformed brain in comparison to uninjected controls as expected (Fig. 3B). Treating *Kv1.5* mRNA-injected embryos with M50054 (20 μM) resulted in near complete prevention of the effect of *Kv1.5* mRNA injections (Fig. 3B; χ^2 test, $p<0.001$, post test $^{**}p<0.01$). We conclude that V_{mem} regulation of apoptosis within the developing brain tissue is a major contributor to the induction of brain morphology defects resulting from perturbation of V_{mem} patterning, since such defects can be largely prevented by inhibiting apoptosis.

Both local and distant V_{mem} signals regulate proliferation in the developing brain

In addition to apoptosis, proliferation is a major factor regulating tissue boundaries and organ size during development and regeneration (Chan et al., 2002, Joseph and Hermanson, 2010, Shitamukai and Matsuzaki, 2012, Stanger, 2008a, Stanger, 2008b). Our previous study showed that V_{mem} affects proliferation in the developing brain (Pai et al., 2015), as it does in a number of normal and neoplastic tissues (Blackiston et al., 2011, Blackiston et al., 2009, Chernet and Levin, 2013a, Chernet and Levin, 2014, Ding et al., 2012, Higashimori and Sontheimer, 2007, Nilius and Wohlrab, 1992, Yang and Brackenbury, 2013, Zhang et al., 2012). Hence, we next analyzed proliferation within the developing brain of *Kv1.5* (hyperpolarizing) mRNA injected *Xenopus* embryos by immunostaining transverse sections through brain, for phosphorylated histone 3B (H3P; a proliferation marker; (Saka and Smith, 2001, Sanchez Alvarado, 2003). Sections anterior to, or through, the eye were used for analysis of developing brain tissue. Control (uninjected) embryos show H3P signal mainly adjacent to the neural canal (Fig. 4Ai). This observation is consistent with previous reports identifying this region as a niche for pluripotent cells from which neural progenitor cells are generated (Chan et al., 2002, Gotz and Huttner, 2005, Stanger, 2008a, Stanger, 2008b). Embryos injected with *Kv1.5* (hyperpolarizing) mRNA into the dorsal two cells at four-cell stage (for targeting neural tissues) significantly decreased the H3P signal in the embryonic

brain in comparison to the controls (Fig. 4Aiii, B; n>11 embryos for each experimental group, one-way ANOVA, p<0.001, with post-tests). We verified our targeting of dorsal tissue by co-injection of β-*galactosidase* mRNA along with *Kv1.5* (hyperpolarizing) mRNA and then performing the H3P immunostaining on sections of β-galactosidase-stained embryos (Fig. 4Cii). Injecting *Kv1.5* (hyperpolarizing) mRNA in only one dorsal blastomere slightly (non-significantly) decreased H3P signal in the developing brain in comparison to controls (Fig. 4Aii). Interestingly, *Kv1.5* (hyperpolarizing) mRNA injections in the ventral two blastomeres at four-cell stage, significantly increased H3P staining in the developing brain and eye in comparison to controls (Fig. 4Aiv, B and Ci), confirming the long distance action of ventral V_{mem} patterns in upregulating proliferation in the developing brain, and once again ruling out nonspecific toxicity as an explanation of the consequences channel-induced voltage change. Dorsal injections decreased the proliferation, consistent with the known hyperpolarized state of quiescent (non-proliferative) cells (Binggeli and Weinstein, 1986a, Blackiston et al., 2009). Remarkably, *Kv1.5* (hyperpolarizing) mRNA injections into both dorsal and ventral blastomeres were not intermediate between the high ventral and low dorsal outcomes, but were even higher than ventral alone. Injection into both dorsal and ventral regions induced significantly increased H3P signal in the developing brain and eye (Fig. 4Av and B) suggesting a predominant effect of the ventral V_{mem} patterns on H3P signal within the developing brain.

The most striking was the effect of one-sided injections (left two blastomeres at four-cell stage), after which H3P signal within the brain significantly increased on *both* the injected (β-galactosidase positive) and the contralateral uninjected (β-galactosidase negative) side, revealing that ventral V_{mem} state signaling crosses the midline in regulating proliferation (Fig. 4Ciii). These results suggest that dorsal V_{mem} patterns act locally, reducing proliferation with the developing brain tissue. Contrarily, the ventral V_{mem} patterns act at a distance, enhancing the proliferation within the developing brain tissue. The results suggest that the ventral V_{mem} pattern's effect on proliferation within the developing brain tissue is dominant, and underline the long-range nature of bioelectric signaling which crosses both the dorso-ventral and also the left-right axes.

Spinal cord apoptosis is regulated only by local V_{mem} signal and proliferation only by long-distance V_{mem} signal

Do V_{mem} patterns play a similar role in other aspects of CNS, particularly spinal cord development? To assess this, we analyzed apoptosis and proliferation (as described in detail above) within the developing spinal cord of V_{mem}-perturbed (hyperpolarized by *Kv1.5* mRNA injections) *Xenopus* embryos by immunostaining the transverse sections through the developing spinal cord, for activated caspase 3 and H3P respectively. Sections posterior to the otic/ear vesicles and through the yolk were used for analysis of developing spinal cord. In control (uninjected) embryos active caspase 3 was intermittently and sparsely distributed within the nerve cord similar to brain tissue (Fig. 5A and Ci). Embryos injected with *Kv1.5* (hyperpolarizing) mRNA into the dorsal two cells at four-cell stage (for targeting neural tissues) showed significantly increased active caspase 3 signal within the embryonic spinal cord in comparison to controls (Fig. 5A and Cii; n>10 for each experimental group, one-way ANOVA, p<0.001, with post-tests). Contrary to the observations in the brain tissue, *Kv1.5*

(hyperpolarizing) mRNA injections in the ventral two blastomeres had no effect on active caspase 3 signal (Fig. 5A and Ciii). Analogously, *Kv1.5* (hyperpolarizing) mRNA injections in both the dorsal and ventral blastomeres showed no change in the active caspase 3 signal in comparison to the dorsal blastomere-injected embryos, which exhibited the expected increase in apoptosis (Fig. 5A and Civ). These results suggest that although the local (dorsal) V_{mem} patterns regulate apoptosis in the developing spinal cord similar to that in the developing brain, the ventral V_{mem} pattern has no bearing on regulation of apoptosis in the developing spinal cord unlike within the developing brain.

When assessed for proliferation, in control (uninjected) embryos the H3P signal was mainly present adjacent to the neural canal of the developing spinal cord, similar to the developing brain pattern (Fig. 5B and Cv). *Kv1.5* (hyperpolarizing) mRNA injection into the dorsal two cells at four-cell stage (for targeting neural tissue) showed no change in the H3P signal within the developing spinal cord in comparison to controls (Fig. 5B and Cvi; $n>8$ for each experimental group, one-way ANOVA, $p<0.001$, with post-tests). *Kv1.5* (hyperpolarizing) mRNA injections into the ventral two blastomeres (targeting non-neural tissue) significantly increased H3P signal in the developing spinal cord in comparison to controls, similar to the effect seen in the developing brain (Fig. 5B and Cvii). *Kv1.5* (hyperpolarizing) injections in both the dorsal and ventral blastomeres at four-cell stage had unchanged levels of H3P signal in comparison to the dorsal two blastomeres injected embryos (Fig. 5B and Cviii). These results suggest that although the long distance (ventral) V_{mem} patterns regulate proliferation within the developing spinal cord similar to the developing brain, the dorsal (local) V_{mem} pattern has no bearing on regulation of proliferation in the developing spinal cord unlike the developing brain.

These data reveal a simpler regulation of apoptosis (by local dorsal V_{mem} patterns) and proliferation (by distant ventral V_{mem} pattern) within the developing spinal cord in comparison to the developing brain. We conclude that the anterior and posterior aspects of the CNS both utilize bioelectric signaling but in distinct spatial modes.

Discussion

Membrane voltage potential signals over long-distance impinge upon developmental brain morphology

Dynamic changes in resting membrane potentials (V_{mem}) carry patterning information during embryonic organ development in *Xenopus* (Adams and Levin, 2013, Levin, 2014a, Mustard and Levin, 2014, Tseng and Levin, 2013b, Vandenberg et al., 2011). In addition to activity-dependent sculpting of neural connections (Kozorovitskiy et al., 2012, Penn and Shatz, 1999), the spatial distribution patterns of V_{mem} in developing embryos controls aspects of large-scale morphogenesis of the nervous system, particularly eye and brain (Beane et al., 2013, Pai et al., 2015, Pai et al., 2012a, Pai et al., 2012b). Here we assess the effect of V_{mem} specifically on apoptosis and proliferation within the developing central nervous system (CNS) as it regulates the large-scale morphogenesis of the brain and spinal cord. Particularly we examine the effect of local and long-distance V_{mem} signals in regulating these cell processes.

Molecularly and spatially regulating V_{mem} with microinjections of well-characterized ion channel mRNAs is a very tractable and well established experimental method (Adams and Levin, 2013). It erases the endogenous differential spatial distribution patterns of V_{mem} of neural and non-neural tissues that encode patterning information necessary proper brain morphogenesis (Pai et al., 2015). While here we focused our analysis on the most potent hyperpolarizer, Kv1.5, our previous work showed the effect is truly voltage-dependent, and not tied to a specific ion channel protein, as many other ion translocators with the same effect on V_{mem} can be substituted (Pai et al., 2015, Pai et al., 2012a).

Although absolute V_{mem} is known to control cell proliferation and differentiation (Blackiston et al., 2009, Sundelacruz et al., 2008, Sundelacruz et al., 2009, Sundelacruz et al., 2013), the effect of relative V_{mem} differences between groups of cells on cellular and tissue processes is only beginning to be understood. Cell autonomous local bioelectric signaling has been observed in eye (Pai et al., 2012a) and brain (Pai et al., 2015) patterning, and is mainly transduced by intracellular calcium signaling and GJCs to regulate cellular behavior. Non-cell-autonomous bioelectric signaling has been observed in brain development (Pai et al., 2015), tumor suppression (Chernet et al., 2014), ectopic innervation (Blackiston et al., 2015a), and left-right patterning (Levin and Mercola, 1999). While the transduction mechanisms of such effects are beginning to be understood, the extent of such signaling during normal development has not been explored. Understanding this interplay between these local and long-distance bioelectric transduction mechanism is critical in understanding topology of signaling resulting in neural development.

This study attempts to tease apart contributions of relative V_{mem} levels during normal development of CNS. Altering or erasing the characteristic local hyperpolarization pattern within the developing neural tube leads to defects in brain patterning ranging from small or absent nostrils and forebrain, deformed or shrunken midbrain and deformed eyes, while the rest of the animal developed normally [Fig. 1 and (Pai et al., 2015, Pai et al., 2012a)]. Hence the local V_{mem} pattern seems to function as a distinct bioelectric signal impinging on brain patterning (Pai et al., 2015, Pai et al., 2012a). However, a near complete suppression or rescue of this phenotype is achieved by hyperpolarizing the distant (ventral) region (which directly does not contribute to neural development) (Fig. 1), suggesting distant V_{mem} patterns from surrounding tissues also potentially impinge on brain patterning. These results suggests that during embryonic development, at least in brain patterning, information from both local and distant bioelectric patterns is read and incorporated towards correct morphological patterning of the brain tissue.

Local and distant V_{mem} patterns regulate apoptosis and proliferation within the developing brain

Disrupting or erasing the local (dorsal, neural) endogenous V_{mem} gradient pattern within the developing neural tube increased apoptosis (Fig. 2) and decreased proliferation (Fig. 4) within the developing brain and eye. Inhibiting apoptosis with chemical inhibitors reduced the ability of V_{mem} disruption to cause brain mispatterning (Fig. 3). These results suggest that local (dorsal, neural) bioelectric signals' regulation of apoptosis is an important endogenous component of proper brain tissue morphology during development (Fig. 1); this

effect is in addition to voltage regulation of proliferation. Perturbing distant (ventral, non-neural) bioelectric patterns decreased apoptosis (Fig. 2) and strongly increased proliferation (Fig. 4) within the developing brain. Perturbing both distant (ventral, non-neural) and local bioelectric states resulted in reduced apoptosis in comparison to perturbation of local bioelectric states alone (Fig. 2). In case of proliferation, the distant (ventral, non-neural) bioelectric cell states were able to completely override the effect of local bioelectric signal perturbation (Fig. 4). These results suggest that both local (dorsal, neural) and distant (ventral, non-neural) bioelectric signals regulate cellular behavior (apoptosis and proliferation) within the developing brain, in opposite directions. In case of apoptosis, since the effect of perturbing distant bioelectric signals only partially reverse the effect of local bioelectric signal (Fig. 2), it can be postulated that the local (dorsal, neural) bioelectric signals are the dominant of the two. Contrarily, in case of proliferation, since effect of perturbing the distant bioelectric signal overrides the effect of local bioelectric signals on proliferation, it suggests the distant (ventral, non-neural) bioelectric signals are the dominant of the two.

Although perturbing local (dorsal, neural) bioelectric signals increases apoptosis in the developing brain, it cannot be attributed to toxicity or merely disruption of housekeeping processes since perturbing both local and distant (ventral, non-neural) bioelectric signal actually decreases apoptosis in the brain. Instead it suggests instructive signaling by bioelectric signals as shown for bioelectric signals in other contexts (Konig et al., 2004, Ng et al., 2010, Pai et al., 2015, Pai et al., 2012a, Pai et al., 2012b, Sundelacruz et al., 2009, van Vliet et al., 2010). Further, it suggests integration of information from more than one bioelectric signal (local and distant) converging towards regulation of particular cell/tissue process during embryonic organ patterning.

Apart from developing brain, bioelectric signal also regulate apoptosis in region around the developing brain particularly in cells surrounding the notochord (not within the notochord) (Fig. 2). Notochord and notochord-derived signals are absolutely crucial in induction of the neural plate, formation of neural tube and differentiation of regions of neural tube (Altmann and Brivanlou, 2001). During embryonic development such inductive interactions between germ layers and between tissues are critical in achieving target morphologies of organs (Tannahill et al., 2005). Apoptosis in these cells around the notochord and the developing brain could disrupt such critical inductive interactions by either eliminating signal producing cells or signal receiving cells. Such secondary effect of apoptosis on inductive interactions during brain development have previously been documented (Nonomura et al., 2013). Hence, this apoptosis in the regions around notochord and developing brain may also be responsible for the observed defects in brain morphology upon perturbation of bioelectric signals. The specific nature of the inductive interactions that may be disrupted by this bioelectric signal-regulated apoptosis and how they affect embryonic brain morphology and patterning remains an area of active investigation.

An interesting observation in bioelectric regulation of apoptosis within the developing brain is that the distant (ventral, non-neural) bioelectric signals regulate apoptosis in the developing brain across the dorso-ventral axis, however, the local (dorsal, neural) bioelectric signal is highly localized and does not act across the midline from the left to right side of

the developing neural tissue (Fig. 2). Unlike the predominant (local) bioelectric regulation of apoptosis which does not cross the midline and is highly local, the predominant (distant) bioelectric regulation of proliferation functions across the dorso-ventral as well as the midline (left-right) axis (Fig. 4). How is such tight spatial control achieved between the local and distant bioelectric signals? And more important what is the evolutionary significance or advantage of such spatially differential control of apoptosis and proliferation by bioelectrical signals in generating the target morphology of the embryonic brain? These aspects remain to be probed in subsequent studies.

Together, these observations reveal the important interplay between the local and distant bioelectric states in regulating apoptosis and proliferation within the developing embryonic brain. Patterning information from both types of bioelectric signals is integrated towards generating proper brain morphology in the developing embryo.

Local and distant V_{mem} patterns have counterbalancing effects on apoptosis and proliferation within the developing brain

Both apoptosis and proliferation within the developing brain have at least two controls: local bioelectric signal and distant bioelectric signal. Interestingly, these two bioelectric controls have opposite actions (one decreases, other increases) on the same process (proliferation or apoptosis) (Fig. 2 and 4). Such coupling of two bioelectric counter controls converging on one cellular process (in this case proliferation or apoptosis) result in a system that is able to finely tune the extent of that cellular process to meet the morphological and physiological requirements of the developing embryo. Interestingly, such coupling of opposite processes is seen at a higher level of complexity with the local bioelectric signals being predominant in regulating apoptosis whereas the distant bioelectric signals being predominant in regulating proliferation within the same tissue (developing brain). Such dueling controls could be important in regulating the size of a tissues/organs in relation to the size of embryo/organism as a whole, by coupling the dynamic morphology of growing structures to the physiological state of surrounding (or even distant) tissues *in vivo*.

Bioelectric signal regulate apoptosis and proliferation within the developing brain

What could be the mechanism employed by the bioelectrical signals towards regulation of apoptosis and proliferation in the embryonic brain? There are several possibilities, currently under investigation.

It is possible that perturbing the distant bioelectric signals results in mispatterning of ventral components of the neural structures (such as notochord) that play a role in inductive interactions resulting in formation of the neural plate, neural tube and the brain (Altmann and Brivanlou, 2001, Tannahill et al., 2005). However, this hypothesis is less likely, as in such a situation at least some incidence of mispatterned ventral tissues would occur, which we do not see in our experimental groups (notochord, gut and endodermal structures all form normally). Another possible mechanism of action of distant bioelectric signals could be through regulation of morphogen production/response in the inductive tissues like the notochord (Tannahill et al., 2005). Notochord releases morphogens like serotonin (5-HT) and sonic hedgehog (Shh) which regulate apoptosis, proliferation, and differentiation within

the embryonic developing brain (Lauder et al., 1981, Tannahill et al., 2005). Bioelectric signal-mediated regulation of morphogen synthesis and signaling has been observed at the transcriptional level in *Xenopus* embryos (Pai et al., in review). Thus notochord and its morphogens, may act as a sensor or integrator system of the bioelectric signals (both local and distant) towards appropriate embryonic brain patterning. Interestingly, notochord is formed before neural tissue specification and is located at the nexus of dorsal-ventral and left-right body axis, an ideal location for a sensor/integrator.

Previous studies have shown a critical role of gap junction channels (GJCs) in transducing the effect of distant bioelectric signal perturbations on proliferation within the developing brain (Pai et al., 2015). Movement of serotonin through GJCs has also been shown to be an important component of distant bioelectric communication guiding ectopic nerve growth (Blackiston et al., 2015a). Finally, modeling and experimental testing of model predictions has revealed that the left and right sides of the *Xenopus* embryo produce a coherent long-range bioelectric growth-control signal that is coordinated by the action of GJCs (Chernet et al., 2014). Disrupting GJC communication on one side of the embryo affected V_{mem}-driven physiological changes (rate of tumor formation in oncogene-injected embryos) on the other side (Chernet et al., 2014). A similar GJCs-mediated mechanism could also be responsible for transducing the distant bioelectric signals regulating apoptosis within the developing brain. Alternatively, GJCs could serve as conduits for transducing bioelectric patterning information to sensor/integrators (like notochord as discussed above) tissues which in turn regulate the levels of apoptosis and proliferation in the developing brain. Cell to cell gap junction connections are quite dynamic during embryonic development (Anava et al., 2013, Warner, 1985), and the sensitivity of GJs to transjunctional voltage (Palacios-Prado and Bukauskas, 2009, Verselis et al., 1991) suggest gap junctions as the ideal candidate to mediate cells' ability to compare their voltage with neighboring cell groups. The molecular identity of the signal (ionic, biochemical, or both) passing through GJCs communicating this information is not yet known, although prior work suggests neurotransmitters such as serotonin as a likely candidate for subsequent analysis (Blackiston et al., 2011, Blackiston et al., 2015b, Fukumoto et al., 2005, Lobikin et al., 2012a). Understanding the precise transduction mechanism of local and distant bioelectric signals in regulating brain patterning remains an active area of investigation.

Bioelectric signal regulate apoptosis and proliferation in embryonic spinal cord development

The bioelectric regulation of apoptosis and proliferation in the developing spinal cord appears to be simpler with less regulation than in the developing brain. Apoptosis in the developing spinal cord is regulated only by the local bioelectric signal with no detectable input from distant bioelectric signals on apoptosis (Fig. 5). In contrast, proliferation in the developing spinal cord is regulated only by the distant bioelectric signal with no detectable input from the local bioelectric signals on proliferation at the relevant developmental period (Fig. 5). Moreover, in comparison to brain development, the dominant bioelectric signals (local for apoptosis and distant for proliferation) seem to persist but the subtler/weaker bioelectric signals (distant for apoptosis and local for proliferation) seem to be absent.

Why is the bioelectric signal regulation of apoptosis and proliferation in embryonic spinal cord development simplified in comparison to the developing brain is not yet known. The brain has been postulated to have evolved from the cephalization (bulging or growing) of the anterior end of the neural tube/spinal cord of cephalochordates like Amphioxus (Northcutt, 1996, Northcutt, 2003). Could the subtle bioelectric controls seen in brain development but absent in spinal cord development be a part of brain evolutionary mechanism from rudimentary neural tube/spinal cord? This hypothesis will be addressed in future work.

A model integrating the bioelectrical signals in regulating apoptosis and proliferation in brain development

We suggest a model integrating the collective data on bioelectric signals controlling apoptosis and proliferation during brain patterning from this and previous study (Fig. 6). The instructive bioelectric signal (V_{mem} state) is physiological in nature and hence not identical with any one gene product (ion channel), as numerous ion translocators contribute to overall V_{mem} and can compensate for one another in setting correct voltage state during pattern formation (Blackiston et al., 2011, Pai et al., 2012a). This aspect is extremely desirable from the perspective of developing therapeutic applications as it enables a broad range of potential reagents and endogenous targets for pharmacological treatment of brain malformation defects.

Disturbing the endogenous local (dorsal, neural) bioelectric pattern leads to increase in apoptosis and decrease in proliferation in the developing brain (Fig. 6ii). The ensuing shift in the apoptosis-proliferation balance towards apoptosis leads to mispatterning of the developing brain (Fig. 6ii). Further, inhibiting apoptosis prevents this shift in balance towards apoptosis and rescues the embryos from the bioelectric mispatterning. Disturbing the endogenous distant (ventral, non-neural) bioelectric signal leads to decrease in apoptosis and increased proliferation with a largely normally patterned brain (Fig. 6iii). While a role of bioelectric signal in regulating apoptosis-proliferation has been shown in determining planarian regeneration (Beane et al., 2013), this is the first account of bioelectric signal (particularly contribution of both local and distant bioelectric signals) controlling the apoptosis-proliferation balance in sculpting the neural tissue in a developing vertebrate embryo.

Conclusion

We have shown that endogenous patterns of bioelectric signals are key determinants of CNS development. The bioelectric signals of local and distant tissues are important endogenous components involved in brain and spinal cord sculpting particularly at the level of regulating apoptosis and cell proliferation. We reveal a complex counter-balancing interaction of local and distant bioelectric cell states in regulating key morphology determining cellular behaviors of apoptosis and proliferation. Taken together, these data show a new function of bioelectric signals in controlling the apoptosis-proliferation balance during embryonic brain and spinal cord patterning. Especially interesting are the long-range rescue effects, which suggest a clear roadmap using ion channel drugs (electroceuticals) targeted to non-neural

tissues as a tractable strategy for manipulating neurogenesis and neural patterning in the context of birth defects, regenerative medicine, and synthetic bioengineering.

Materials and Methods

Animal husbandry

Xenopus laevis embryos were fertilized *in vitro* according to standard protocols (Sive et al., 2000) in 0.1X Marc's Modified Ringer's solution (MMR; 10mM Na^+, 0.2mM K^+, 10.5 mM Cl^-, 0.2 mM Ca^{2+}, pH 7.8). *Xenopus* embryos were housed at 14-18°C (14°C overnight after injection and subsequently at 18°C) and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). PNTub::GFP transgenic *Xenopus* were created as described (Kroll and Amaya, 1996) except that the restriction enzyme was omitted (Lin et al., 2012, Marsh-Armstrong et al., 1999). There is no practical way to precisely determine the sex of the embryos at the stages at which these procedures are done, and the ratio of male:female should be 50:50 in all of our experiments. All experiments were approved by the Tufts University Animal Research Committee (M2014-79) in accordance with the guide for care and use of laboratory animals.

Microinjections

Capped synthetic mRNAs generated using mMessage mMachine kit (Ambion) were dissolved in nuclease free water and injected into embryos immersed in 3% Ficoll using standard methods (Sive et al., 2000). Each injection delivered between ~3 nL or ~3-4 ng of mRNA (per blastomere) into the embryos, usually at 4-cell stage into the middle of one or more cells (in the animal pole) as indicated in the Results section. Constructs used was *Kv1.5* (Strutz-Seebohm et al., 2007) and *Kv1.5-2A-b-galactosidase*, both in vector PCS2. *Kv1.5-2A-b-galactosidase* was injected as a bi-cistronic RNA (with single polyA tail at the end) which produces separate proteins, due to a viral peptide sequence (2A) inserted between the 2 cDNA sequences (de Felipe et al., 2006, Szymczak-Workman et al., 2012). Kv1.5 is a commonly-used hyperpolarizing channel (Bertoli et al., 1994, Pai et al., 2012a, Strutz-Seebohm et al., 2007).

Immunofluorescence and immunohistochemistry

Spatial detection of apoptosis and proliferation was performed by immunofluorescence for activated caspase 3 and phospho-histone H3P respectively, on sections. Stage 30 embryos were used because by this stage, the brain region of the neural tube is specified and the brain morphology is now developing, making it a good stage to assess apoptosis and proliferation within the developing brain. Briefly, embryos were fixed overnight in MEMFA at 4 degrees (Sive et al., 2000), embedded in agarose and sectioned at 100 μm thickness using a Leica vibratome (VT1000S) as per standard protocol (Blackiston et al., 2010) or embedded in paraffin and sectioned at 5 μm thickness using Leica microtome as per the standard protocol (Sive et al., 2000). The sections [> 5 sections per embryo (n = number of embryos as indicated in the results)] were permeabilized in PBS 0.1% Triton X-100 (PBST), antigen retrieved using citrate buffer (pH 6.0) (heating in a microwave), blocked with 10% goat serum in PBST for 1 hour at room temperature and incubated at 4°C overnight with primary antibody (Apoptosis – Anti-Active Caspase 3; Abcam ab13847) (Proliferation

- Anti-H3P; Millipore-Invitrogen 04-817) at 1:1000 dilution in PBST+10% goat serum (blocking buffer). Sections were washed six times in PBST and incubated with Alexa-Fluor conjugated fluorescent secondary antibody (Invitrogen) at 1:500 dilution in PBST + 10% goat serum overnight at 4 °C. Sections were washed six times in PBST and photographed using Olympus BX-61 microscope equipped with a Hamamatsu ORCA AG CCD camera, and controlled by Metamorph software.

Drug exposure

Xenopus embryos were incubated in pharmacological blockers dissolved in 0.1X MMR during the stages of interest as indicated in respective experiments followed by several washes with 0.1X MMR. Embryos were exposed (from stage 10 – stage 30 unless otherwise specified, because neural/brain tissue development takes place in this time period, allowing specific testing of effects on these processes while allowing cleavage and gastrulation to proceed normally) to: 20 μM Apoptosis inhibitor M50054 (CalbioChem 178488).

Beta-galactosidase staining

Embryos injected with β-*gal* mRNA were fixed at gastrula or neurula stages, washed, and stained with X-gal (Roche Applied Sciences, Indianapolis, IN) at 37°C.

Statistics

All statistical analysis was performed using Microsoft Excel. Data was either pooled from various iterations, with χ^2-Square analysis performed on them, or data from various iterations was analyzed by *t*-test (for 2 groups) or ANOVA (for more than two groups).

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Abbreviations used in this paper:

CNS	Central Nervous System
GJCs	gap junctional communication
V_{mem}	transmembrane voltage potential

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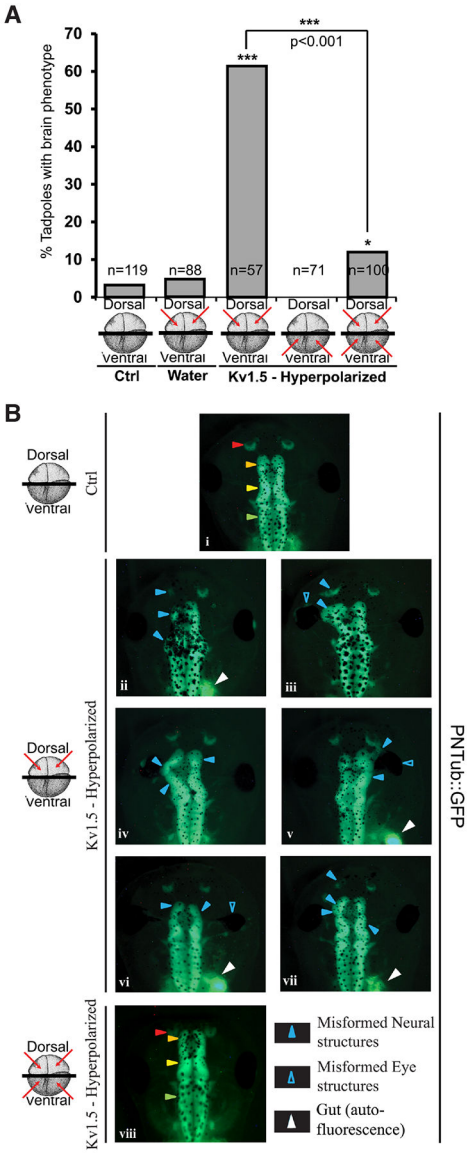

Fig. 1. Kv1.5 mediated local V_{mem} perturbation disrupts endogenous brain development.

(A) *Quantification of tadpoles with brain phenotypes upon microinjections of hyperpolarizing* Kv1.5 *ion channel mRNA in the indicated cells (red arrows) of the four cells* Xenopus *embryo. A high incidence of misformed brain is observed in dorsal injections in comparison to uninjected controls. A* χ^2 *analysis was performed. The dorsally injected embryos were significantly different from the controls (*** p<0.001). Ventral injections were not significantly different from controls. Injections in both dorsal and ventral regions showed decrease in misformed brain in comparison to dorsal injections alone. A t-test showed both dorsally and ventrally co-injected embryos were significantly different from the dorsally injected embryos (***p<0.001).* **(B)** *Control (uninjected) and Hyperpolarizing* Kv1.5 *mRNA injected (red arrows indicate injected cells) in 4-cell PNTub::GFP transgenic* Xenopus *embryos. (i) Stage 45 PNTub::GFP controls show GFP fluorescence in the neural tissue. The uninjected control tadpoles show well-formed anterior neural tissues. Control*

tadpoles show well-formed anterior neural tissue with red arrowheads indicating nostrils, orange arrowheads indicating forebrain/olfactory bulbs, yellow arrowheads indicating mid-brain and green arrowheads indicating hindbrain. (ii-vii) Stage 45 PNTub::GFP transgenic tadpoles dorsally injected with hyperpolarizing channel Kv1.5 *mRNA. Solid Blue arrowheads indicate severely malformed midbrain and forebrain. Empty blue arrows indicate eyes which are also found to be malformed or absent. (viii) Stage 45 PNTub::GFP transgenic tadpoles injected with hyperpolarizing channel* Kv1.5 *mRNA in both dorsal and ventral blastomeres showing brain structure similar to the uninjected controls (i). Gut (white arrowhead) is also autofluorescent in the same spectrum.*

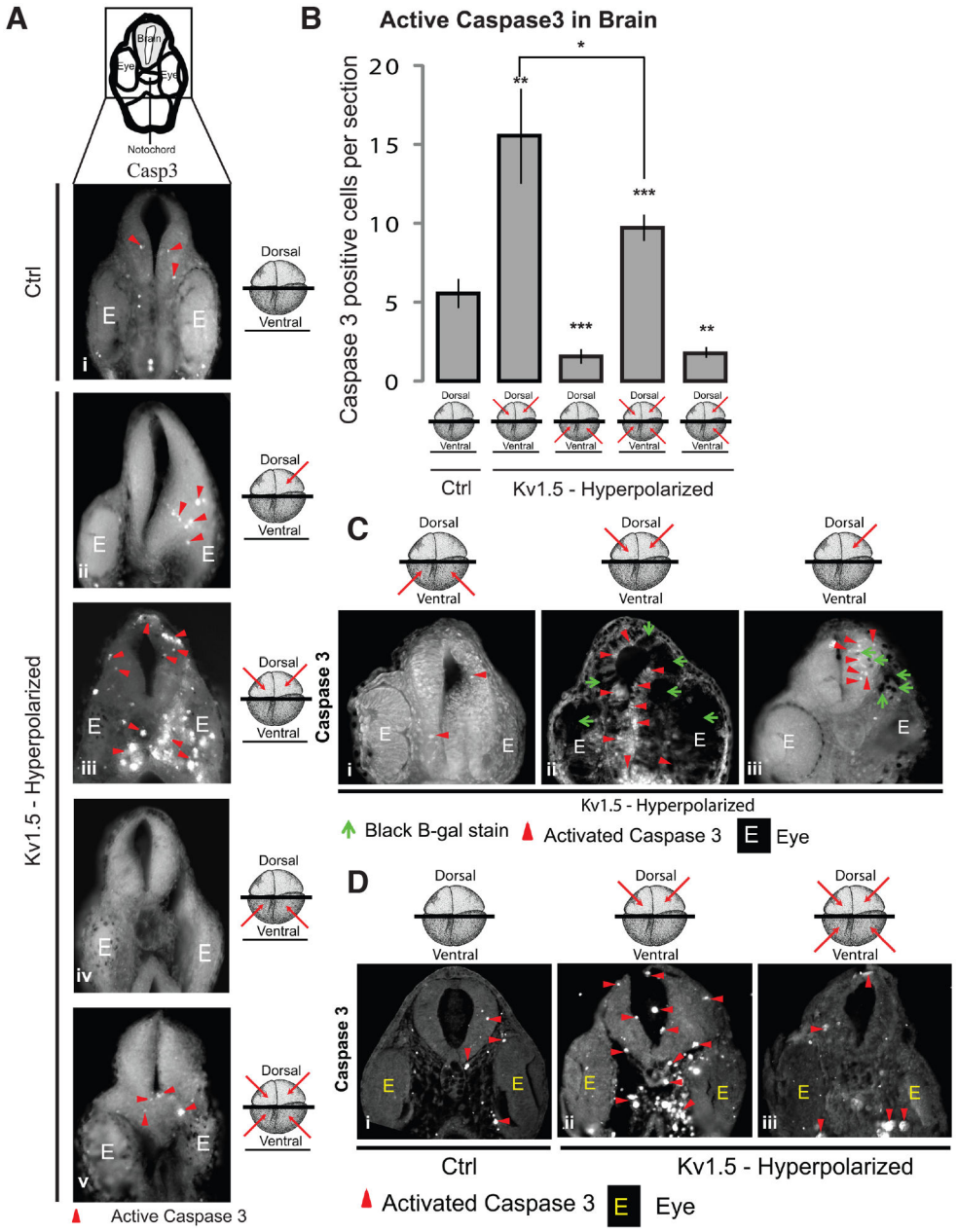

Fig. 2. Both local and distant V_{mem} signals regulate apoptosis in the developing brain.

(A) *Illustration depicts the region of the cross-section shown. Agarose sections of stage 30 control (uninjected) embryos (i) and embryos microinjected with hyperpolarizing* Kv1.5 *mRNA (ii-v) in the indicated blastomeres (red arrows) at 4-cell stage. Immunostainng of sections through the developing brain with activated caspase 3 (i-v) shows a distinct change in the activated caspase 3 (red arrowheads) staining in the developing brain of microinjected embryos in comparison to uninjected controls. E indicates eye tissue.* **(B)** *Quantification of activated caspase 3 immunostaining in the agarose sections through developing brains of stage 30 control (uninjected) and hyperpolarizing* Kv1.5 *microinjected (red arrows indicate injected blastomeres at 4-cell stage) embryos. Dorsal blastomere injections significantly*

*increase the activated caspase 3 signal whereas ventral injections significantly decrease the activated caspase 3 signal. Both dorsal and ventral injections significantly decrease the activated caspase 3 signal in comparison to dorsal only injections. Values are mean + s.e.m. (n>10 for each group). *, p<0.05; **, p<0.01; ***, p<0.001 One way ANOVA with post-test.* **(C)** *Agarose sections of stage 30* Xenopus *embryos co-injected with hyperpolarizing* Kv1.5 *and* β-Galactosidase *lineage tracer mRNA (i-iii) in the indicated blastomeres (red arrows) at 4-cell stage. Green arrowheads show developed* β-*Galactosidase reaction product which is seen as black (injected) regions on the sections. Immunostaining of sections through the developing brain with activated caspase 3 (i-iii) shows a distinct change in the activated caspase 3 (red arrowheads) staining in the developing brain of different microinjected embryos. Dorsal injected embryos show an increase in activated caspase 3 within the developing brain in comparison to ventrally injected embryos. Left dorsal blastomere injection shows an increase in activated caspase 3 only on the injected side in comparison to the contralateral uninjected side.* **(D)** *Paraffin embedded thin sections of stage 30 control (uninjected) embryos (i) and embryos microinjected with hyperpolarizing* Kv1.5 *channel mRNA (ii,iii) in the indicated blastomeres (red arrows) at 4-cell stage.. Immunostainng of sections through the developing brain with activated caspase 3 (i-iii) shows a distinct increase in the activated caspase 3 (red arrowheads) staining in the developing brain and the region around the notochord of dorsally microinjected embryos (ii) in comparison to uninjected controls (i). Both dorsal and ventral injections (iii) shows a decrease in the activated caspase 3 staining in the developing brain and surrounding notochord region in comparison to the dorsally injected embryos (ii).*

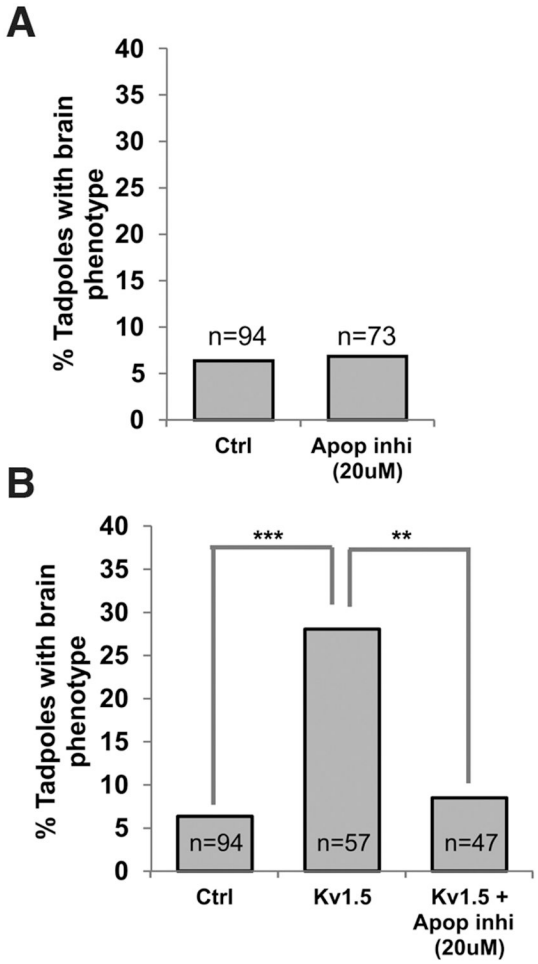

Fig. 3. Inhibition of apoptosis rescues the Kv1.5 induced brain mispatterning.

(A) *Quantification of tadpoles with brain phenotypes in control (untreated) and apoptosis inhibitor treated (M50054 - 20μM) from stage 10 – 30 of* Xenopus *embryos. No significant change was seen on treatment of embryos with the apoptosis inhibitors in comparison to controls. T-test analysis was performed.* **(B)** *Quantification of tadpoles with malformed brain phenotypes in control (uninjected) and hyperpolarizing* Kv1.5 *microinjected (dorsal 2 blastomere at 4-cell stage) embryos with or without the apoptosis inhibitor (M50054-20μM; treated from stage 10-30). A* χ^2 *analysis showed significant variance among the groups. A significantly high incidence of malformed brain phenotype is seen in* Kv1.5 *microinjected embryos (post t-test ***, p<0.001). This effect of* Kv1.5 *is significantly prevented by Apoptosis inhibitor (M50054 - 20μM) (post t-test, ***, p<0.001).*

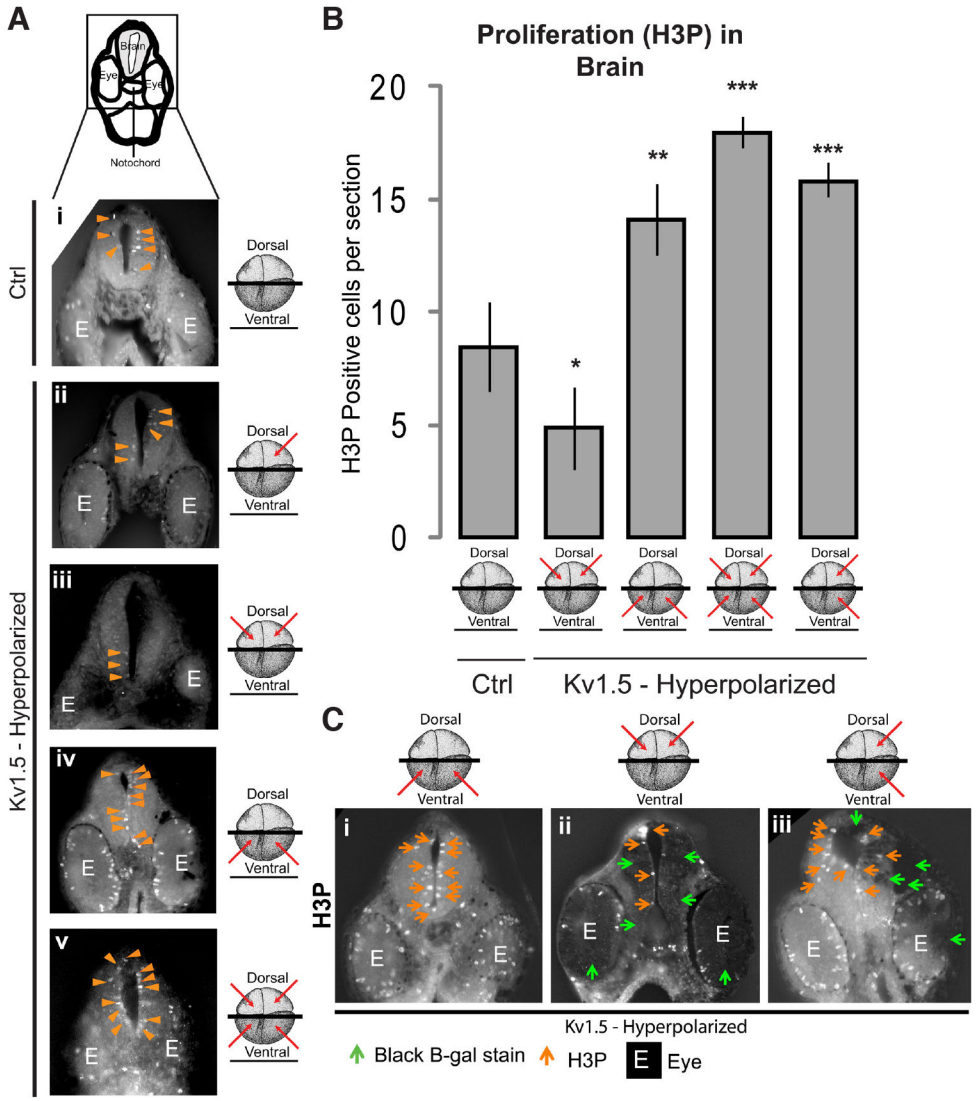

Fig. 4. Both local and distant V_{mem} signals regulate proliferation in the developing brain.

(A) *Illustration depicts the region of cross-section shown. Agarose sections of stage 30 control (uninjected) embryos (i) and embryos microinjected with hyperpolarizing* Kv1.5 *mRNA (ii-v) in the indicated blastomeres (red arrows) at 4-cell stage. Immunostainng of sections through the developing brain with H3P (i-v) shows a distinct change in the H3P (orange arrowheads) staining in the developing brain of microinjected embryos in comparison to uninjected controls.* **(B)** *Quantification of H3P immunostaining in the agarose sections through developing brains of stage 30 control (uninjected) and hyperpolarizing* Kv1.5 *microinjected (red arrows indicate injected blastomeres at 4-cell stage) embryos. Dorsal blastomere injections significantly decrease the H3P signal whereas ventral injections significantly increase the H3P signal. Injections of all 4 blastomeres significantly increases the H3P signal in comparison to dorsal only injections. Values are mean + s.e.m. (n>8 for each group). *, p<0.05; **, p<0.01; ***, p<0.001 One way ANOVA with post-test.*

(C) *Agarose sections of stage 30* Xenopus *embryos co-injected with hyperpolarizing* Kv1.5 *and* β-Galactosidase *lineage tracer mRNA (i-iii) in the indicated blastomeres (red arrows)*

at 4-cell stage. Green arrowheads show developed β-Galactosidase stain which is seen as black (injected) regions on the sections. Immunostaining of sections through the developing brain with H3P (i-iii) shows a distinct change in the H3P (orange arrows) staining in the developing brain of different microinjected embryos. Dorsal injected embryos (ii) show a decrease in H3P within the developing brain in comparison to ventrally injected embryos (i). Left two blastomere injection (iii) shows an increase in H3P (orange arrows) on both the injected (green arrows) and the contralateral uninjected side.

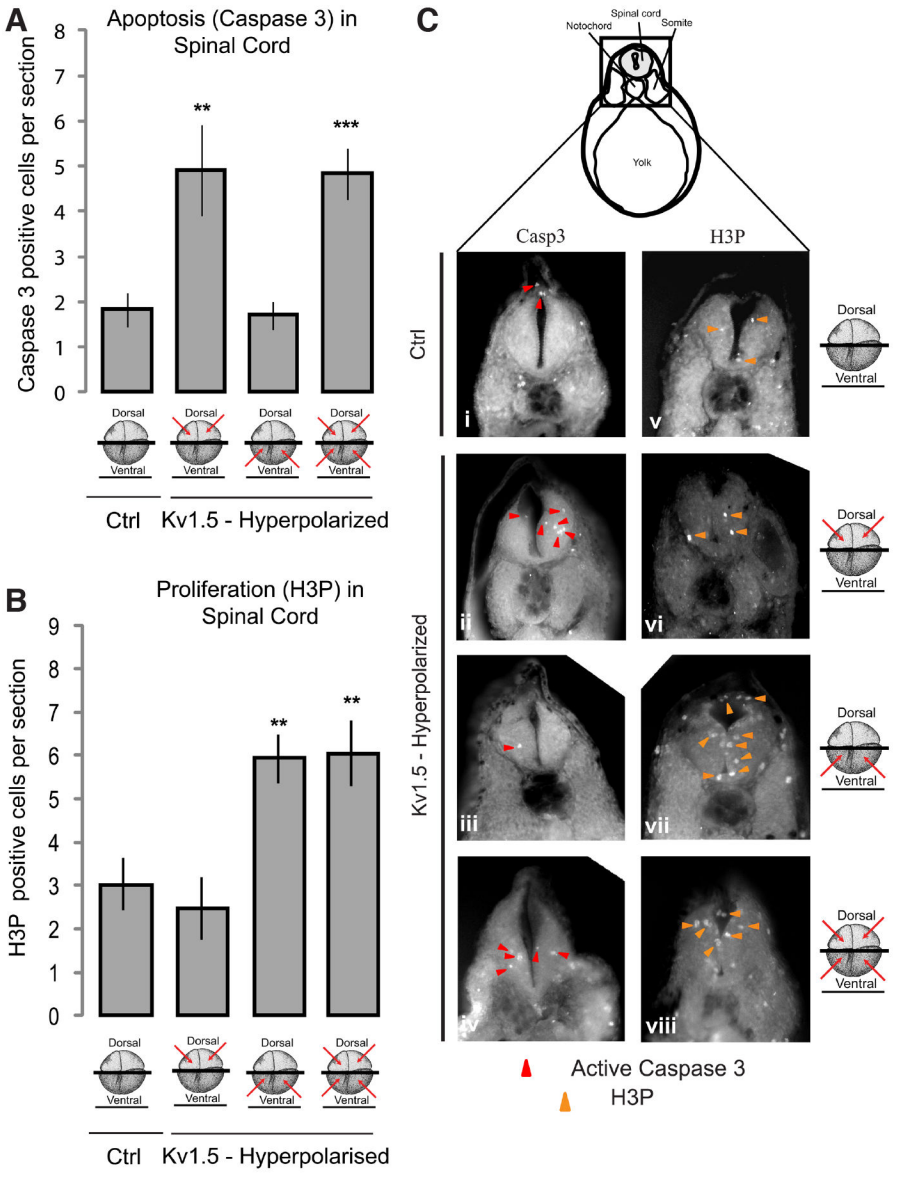

Fig. 5. Both local and distant V_{mem} signals regulate apoptosis and proliferation in the developing spinal cord.

(A) *Quantification of activated caspase 3 immunostaining in the agarose sections through developing spinal cord of stage 30 control (uninjected) and hyperpolarizing* Kv1.5 *microinjected (red arrows indicate injected blastomeres at 4-cell stage) embryos. Dorsal blastomere injections significantly increase the activated caspase 3 signal whereas ventral injections show no change in activated caspase 3 signal. Both dorsal and ventral injections significantly increase the activated caspase 3 signal, similar to dorsal only injections, in comparison to controls. Values are mean + s.e.m. (n>10 for each group). **, p<0.01; ***, p<0.001 One way ANOVA with post-test.* **(B)** *Quantification of H3P immunostaining in the agarose sections through developing spinal cord of stage 30 control (uninjected) and hyperpolarizing* Kv1.5 *microinjected (red arrows indicate injected blastomeres at 4-cell stage) embryos. Dorsal blastomere injections slightly decrease the H3P signal whereas*

*ventral injections significantly increase the H3P signal. Both dorsal and ventral injections significantly increase the H3P signal in comparison to dorsal only injections. Values are mean + s.e.m. (n>8 for each group). **, p<0.01 One way ANOVA with post-test.* **(C)** *Illustration depicts the region of the cross-section shown. Agarose sections of stage 30 control (uninjected) embryos (i and v) and embryos microinjected with hyperpolarizing* Kv1.5 *mRNA (ii-iv and vi-viii) in the indicated blastomeres (red arrows) at 4-cell stage. Immunostaining of sections through the developing spinal cord with activated caspase 3 (ii-iv) shows a distinct change in the activated caspase 3 (red arrowheads) staining in the developing spinal cord of microinjected embryos in comparison to uninjected controls and ventral injections. Immunostaining of sections through the developing spinal cord with H3P (vi-viii) shows a distinct change in the activated caspase 3 (orange arrowheads) staining in the developing spinal cord of microinjected embryos in comparison to uninjected controls and dorsal injections.*

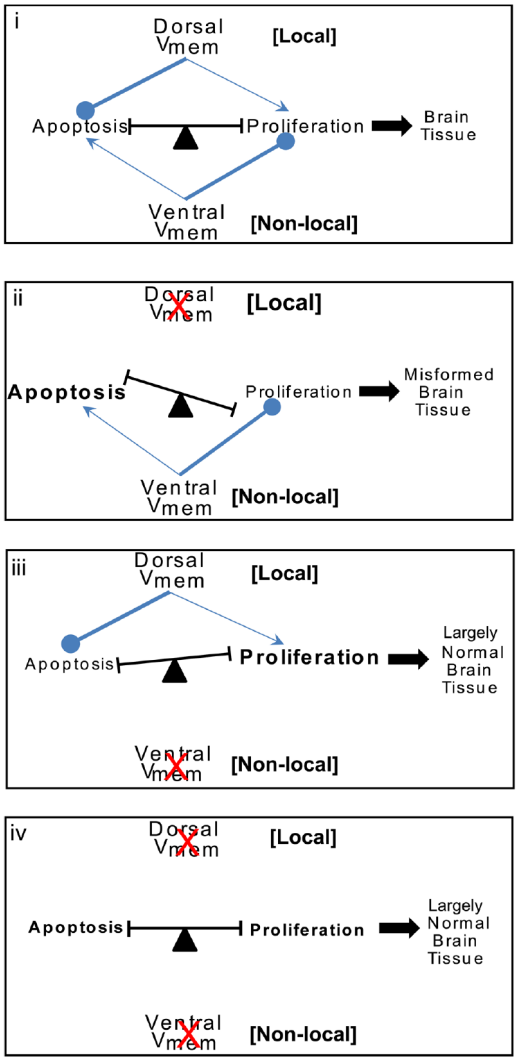

Fig. 6. A model for V_{mem} regulation of apoptosis-proliferation balance towards shaping brain morphology.

Model for V_{mem} regulation of brain tissue sculpting. Both dorsal and ventral V_{mem} signals are involved in brain tissue sculpting by their effects on the balance of apoptosis and proliferation within the tissue. Under normal conditions (i) the dorsal V_{mem} strongly inhibits apoptosis and mildly stimulates proliferation locally within the developing brain. The ventral V_{mem} strongly inhibits proliferation and mildly stimulates apoptosis at a distance in the developing brain tissue. Coordination of these two V_{mem} signals results in proper balance of proliferation-apoptosis and ultimately sculpting of the brain tissue during development. Blocking of dorsal V_{mem} signal (ii) releases the strong inhibition on apoptosis and further facilitates strong inhibition of proliferation by the ventral V_{mem} signals. This results in a shift in balance towards apoptosis resulting in malformed brain tissue. Blocking of ventral V_{mem} signal (iii) releases the strong inhibition on proliferation and increases the inhibition of apoptosis by the dorsal V_{mem} signal. This results in significant increase in proliferating cells but otherwise largely normal brain structure development. Blocking of both dorsal and ventral V_{mem} signal (iv) cancel each other's effect on proliferation and apoptosis leaving

the sculpting of brain to other regulators of brain structure development resulting in largely normal brain structure.