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Bioelectricity Generated by Endogenous Acetylcholine may be An Essential Factor in Salamander Limb Regeneration

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The regeneration of amputated limbs of salamanders and newts has been studied for over a century, however, the exact mechanism of regeneration remains unclear. Previous studies have shown that acetylcholine is present in abnormal levels during the process of limb regeneration. It has been demonstrated that ligand-gated ion channels will open in response to acetylcholine activity, inducing significant ion fluxes. These movements of ions in turn generate small electric currents, which have been shown to influence cell behavior. We have found that these events correlate well with the observed regeneration patterns at the tissue, cellular, and intra-cellular levels. Therefore, we hypothesize that endogenous electric currents – otherwise known as bioelectricity - generated by acetylcholine activity, is an essential factor in salamander and newt limb regeneration.

Key words: Limb regeneration, Bioelectricity, Acetylcholine, Salamander.

Certain species of animals, such as the salamander, have a unique ability to regenerate severed limbs(Binggeli and Weinstein, 1986; McCusker and Gardiner, 2011; Menger *et al*., 2010; Nacu and Tanaka, 2011). Studies on this regenerative process provide insight into stem cell generation and possibilities of medical application of tissue regeneration in higher vertebrates (Menger *et al*., 2010; Roy and Gatien, 2008; Roy and Levesque, 2006; Song *et al*., 2010). In this paper, we aim to explore an alternative hypothesis that until now have evaded extensive research.

Current understanding of regeneration[1]

Once a salamander has one of its limbs amputated, epithelial cells surrounding the wound migrate to form an epidermal layer. The cells beneath this newly formed epidermis will de-differentiate, forming a regeneration blastema (Brockes, 1997; Hyun *et al*., 2012; Tamura *et al*., 2010; Tweedell, 2010).The blastema cells then proliferate, forming a limb bud from which the new limb will grow(Nye *et al*., 2003). Early studies by Todd, Schotte and Butler have identified that sympathetic nerve innervations had a key role in stimulating limb generation(Oscar E. Schotté, 1944; Satoh *et al*., 2012; Todd, 1823). Their observations were followed by a comprehensive series of experiments by Singer, which showed that not only did denervation of a newt limb prevents it from regenerating, but also that if the conditions of the wound is replicated elsewhere on the organism, a limb can be generated at the desired site(Singer and Craven, 1948; Singer and Mutterperl, 1963). Furthermore, it was found that the impact of nerves in regeneration is quantitative, not qualitative (Singer, 1952).

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Although the exact mechanism between nerve stimulation and limb regeneration is still not clearly understood, recent studies have a strong focus on the molecular basis for regeneration. Brockes suggested that if a molecular factor for the survival and proliferation of the blastema cells exists, the candidate molecule should fulfill four criteria; 1) they should be secreted from the AEC (Apical Epidermal Cap)(Stocum, 2004, 2011) or nerve endings into the blastema;, 2) removal of the AEC or nerves should result in loss of the molecule from the blastema; 3) the molecule should be able to substitute for the AEC or nerves in maintain mitosis and/or promoting regeneration to completion; and 4) selective neutralization of the molecule should abolish its mitogenic effect on blastema cells(Brockes, 1984). Based on Brockes suggestion, extensive work in search for these factors have been done in recent years, and growth factors such as molecules in the Fibroblast Growth Factor family, transferrin, Glial Growth Factor-2, substance P, iron-transport protein…etc have been isolated as candidate molecules(Christensen *et al*., 2001; Dungan *et al*., 2002; Globus and Alles, 1990; Mescher, 1996; Mullen *et al*., 1996; Nilsson *et al*., 1985; Wang *et al*., 2000).

Possible role of neurotrasmitter acetylcholine (ACh)

As a major component of the neurotransmitter system, ACh acts by binding to ACh receptors in the extracellular space (also known as the synaptic cleft). When binding to nicotinic receptors, which are ligand-gated ion channels(Arias, 2010; Jadey and Auerbach, 2012), ACh may cause these channels to open, allowing an influx of charged ions. This influx, when significantly large, can generate a weak electric field, otherwise known as bioelectricity. A recent review by Levin stated that regenerating amphibian limbs exhibits a similar electric current, which decreases as the limb heals and regenerates(Levin, 2009).

Presentation of hypothesis

After amputation of a salamander limb, whether from tips to shoulder, damaged nerve fibres near the wound release ACh. This sudden release and accumulation of the neurotransmitter can activate surrounding ligand-gated ion channels, which in turn generates an electric current via the influx of charged ions. We postulate that this electric current, also known as bioelectricity, plays a key role in spatial signaling and activate cellular processes which ultimately determine the success of limb regeneration.

Supporting evidence

Regenerative processes influenced by bioelectricity

The presence of electric currents in living organisms is a phenomenon that has been observed for centuries; these currents have been known to influence regeneration at the tissue, cellular, and intracellular levels. At the tissue level, animals capable of regenerating complex structures produce a direct-current signal can be detected

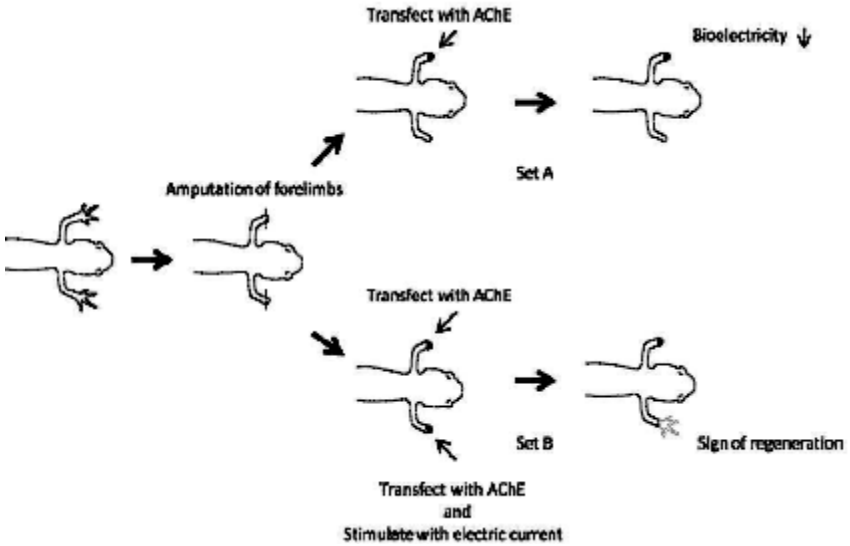

Fig. 1. Diagram showing proposed experimental procedures

during the regenerative process(Borgens *et al*., 1984). Surprisingly, some animals that normally don't exhibit regeneration abilities have successfully regenerated, at least in part, some of the loss structure when an exogenous electric current, similar to that of a regenerative bioelectricity, is applied to amputated structures(Sharma and Niazi, 1990; Smith, 1967; Smith, 1981). Furthermore, the inhibition or interfering (e.g. induced polarity reversal) of these currents leaving amputated limbs of certain regenerative animals disrupts their re-growth (Jenkins *et al*., 1996). It has been suggested that this phenomenon is due to a steady-state electric field that exists in intact tissues and organs, and once the equilibrium is interrupted (e.g. by a flesh wound on the skin), the surrounding cells would react accordingly to inducing healing or regeneration. From this we can see that bioelectricity is an indispensible element of regeneration.

At the cellular level, bioelectricity is essential for cell proliferation, cell differentiation/ de-differentiation, and cell migration(Levin, 2009, 2012; Levin and Stevenson, 2012; McCaig *et al*., 2009). One of the earliest effects of bioelectricity observed is the change of cell orientation and cell migration towards the anode or cathode (Bellamy, 1922). In order to complete limb regeneration, cells must migrate to their pre-determined location to replace lost structures; this is in part guided by electrical gradient generated from the severed nerves, forming a spatial map for cells within seconds of amputation(Shi and Borgens, 1995). In terms of cell differentiation/de-differentiation, Barth and co-workers have shown that it can be modulated by ion-gradients, typically produced when bioelectricity is present(Barth and Barth, 1974). By permitting terminally differentiated cells to de-differentiate, the rate of mitosis is greatly increased, leading to significant proliferation in regenerative tissue(Binggeli and Weinstein, 1986; MacFarlane and Sontheimer, 2000).

At the intra-cellular level, it has been shown that cells important for wound healing, such as keratinocytes and neutrophils, respond to electrical stimulation in serum-free medium(Zhao *et al*., 2006). In Zhao and his colleagues' work, they've found that small electric fields induce rapid and sustained phosphorylation of extracellular-signal-regulated kinase (ERK), p38 mitogen-activated kinase (MAPK), Src, and Akt on Ser 473. These kinase pathways are similar to those induced by chemotactic signaling(Funamoto *et al*., 2002; Kimmel and Parent, 2003; Servant *et al*., 2000), confirming that bioelectricity has a predominant role in tissue regeneration.

Endogenous generation of bioelectricity via ACh

In newts and salamander, ACh level rises significantly after limb amputation, and reaches a maximum level in around 2 weeks(Singer, 1960b). This rise in ACh causes rapid opening of surrounding ion-channels, generating a constant electric current, the residue of which can be measured for weeks to months, much longer than required for normal healing of damaged cells(Becker, 1961; Borgens *et al*., 1984). The change in electrical pattern coincides with the morphogenesis of the lost limb, with the peak voltage being observed when cell proliferation is at a maximum. As regeneration occurs, the amount of ACh is regulated via an increase in acetylcholinase (AChE) activity, gradually decreasing until the completion of morphogenesis, when it returns to normal(Singer, 1960b). Furthermore, in an earlier work studying the regeneration of newt limbs, significant amounts of ACh was found in all stages of limb growth(Singer, 1959), and that blocking agents of ACh such as procaine hydrochloride, atropine sulphate, and tetra-ethylammonium hydroxide exerts a suppression effect on regeneration(Singer, 1960a).

Testing the hypothesis

To test our hypothesis, we propose an experiment in which the level of ACh and electrical current in salamanders' regenerating limbs is controlled.

The salamanders will be divided into two sets of test subjects. The first set of test subjects (Set A) will have their forelimbs amputated mid-way between shoulder and digits, and have their left forelimbs transfected with AChE. Their forelimbs will be tested for the presence and level of bioelectricity daily over the first week, and then weekly over the next 21 days.

The second set of test subjects (Set B) will also have their forelimbs amputated mid-way between shoulder and digits, but will have both forelimbs tranfected with AChE. The right forelimbs will be attached to an electricity source

providing a small current similar to that of endogenous bioelectricity, while the left forelimbs will be untreated. These subjects will be monitored over four weeks and observed for any signs of limb regeneration.

If our hypothesis is confirmed, we would expect that sustained electrical currents can be detected in the right forelimbs of Set A, and the currents detected should be significantly higher than that observed in their left forelimbs, which indicate the importance of ACh's role in bioelectricity generation.

We would also expect the left forelimbs of Set B to show signs of limb regeneration as normal, while their right forelimbs ability to regenerate would be significantly impaired, thus demonstrating the vital role of bioelectricity in limb regeneration.

Conflict of interests

I, Fulin Chen of Northwest University, declare on behalf of all co-authors of "*Bioelectricity generated by endogenous acetylcholine may be an essential factor in salamander limb regeneration*" that no conflicts of interest exist for this submission.

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REFERENCES

1. Arias, H.R., Positive and negative modulation of nicotinic receptors. *Advances in protein chemistry and structural biology*. 2010; **80**: 153-203.
2. Barth, L.G., and Barth, L.J., Ionic regulation of embryonic induction and cell differentiation in Rana pipiens. *Developmental biology*. 1974; **39**(1): 1-22.
3. Becker, R.O., The bioelectric factors in amphibian-limb regeneration. *The Journal of bone and joint surgery*. 1961; 43-A643-656.
4. BELLAMY, L.H.H.A.A.W., Studies on the correlation between metabolic gradients, electrical gradients, and galvanotaxis I. *The Biological Bulletin*. 1922; **43**:3313-347.
5. Binggeli, R., and Weinstein, R.C., Membrane potentials and sodium channels: hypotheses for growth regulation and cancer formation based on changes in sodium channels and gap junctions. *Journal of theoretical biology*. 1986; **123**(4): 377-401.
6. Borgens, R.B., McGinnis, M.E., Vanable, J.W., Jr., and Miles, E.S., Stump currents in regenerating salamanders and newts. *The Journal of experimental zoology*. 1984; **231**(2): 249-256.
7. Brockes, J.P., Mitogenic growth factors and nerve dependence of limb regeneration. Science (New York, NY. 1984; **225**(4668): 1280-1287.
8. Brockes, J.P., Amphibian limb regeneration: rebuilding a complex structure. Science (New York, NY. 1997; **276**(5309): 81-87.
9. Christensen, R.N., Weinstein, M., and Tassava, R.A., Fibroblast growth factors in regenerating limbs of Ambystoma: cloning and semi-quantitative RT-PCR expression studies. *The Journal of experimental Zoology*. 2001; **290**(5): 529-540.
10. Dungan, K.M., Wei, T.Y., Nace, J.D., Poulin, M.L., Chiu, I.M., Lang, J.C., and Tassava, R.A., Expression and biological effect of urodele fibroblast growth factor 1: relationship to limb regeneration. *The Journal of experimental zoology*.2002; **292**(6): 540-554.
11. Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A., Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell*. 2002; **109**(5): 611-623.
12. Globus, M., and Alles, P., A search for immunoreactive substance P and other neural peptides in the limb regenerate of the newt Notophthalmus viridescens. *The Journal of experimental zoology*. 1990; **254**(2): 165-176.
13. Hyun, J.S., Chung, M.T., Wong, V.W., Montoro, D., Longaker, M.T., and Wan, D.C., Rethinking the blastema. *Plastic and reconstructive surgery*. 2012; **129**(5): 1097-1103.
14. Jadey, S., and Auerbach, A., An integrated catch-and-hold mechanism activates nicotinic acetylcholine receptors. *The Journal of general physiology*. 2012; **140**(1): 17-28.
15. Jenkins, L.S., Duerstock, B.S., and Borgens, R.B., Reduction of the current of injury leaving the amputation inhibits limb regeneration in the red spotted newt. *Developmental biology*. 1996; **178**(2): 251-262.
16. Kimmel, A.R., and Parent, C.A., The signal to move: D. discoideum go orienteering. Science (New York, NY. 2003; **300**(5625): 1525-1527.
17. Levin, M., Bioelectric mechanisms in regeneration: Unique aspects and future perspectives. *Seminars in cell & developmental biology*. 2009; **20**(5): 543-556.
18. Levin, M., Molecular bioelectricity in

developmental biology: new tools and recent discoveries: control of cell behavior and pattern formation by transmembrane potential gradients. *Bioessays*. 2012; **34**(3): 205-217.
19. Levin, M., and Stevenson, C.G., Regulation of cell behavior and tissue patterning by bioelectrical signals: challenges and opportunities for biomedical engineering. *Annual review of biomedical engineering*. 2012; **14**: 295-323.
20. MacFarlane, S.N., and Sontheimer, H., Changes in ion channel expression accompany cell cycle progression of spinal cord astrocytes. *Glia*. 2000; **30**(1): 39-48.
21. McCaig, C.D., Song, B., and Rajnicek, A.M., Electrical dimensions in cell science. *Journal of cell science*. 2009; **122**(Pt 23): 4267-4276.
22. McCusker, C., and Gardiner, D.M., The axolotl model for regeneration and aging research: a mini-review. *Gerontology*. 2011; **57**(6): 565-571.
23. Menger, B., Vogt, P.M., Kuhbier, J.W., and Reimers, K., Applying amphibian limb regeneration to human wound healing: a review. *Annals of plastic surgery*. 2010; **65**(5): 504-510.
24. Mescher, A.L., The cellular basis of limb regeneration in urodeles. *The International journal of developmental biology*. 1996; **40**(4): 785-795.
25. Mullen, L.M., Bryant, S.V., Torok, M.A., Blumberg, B., and Gardiner, D.M., Nerve dependency of regeneration: the role of Distal-less and FGF signaling in amphibian limb regeneration. Development (Cambridge, England). 1996; **122**(11): 3487-3497.
26. Nacu, E., and Tanaka, E.M., Limb regeneration: a new development? Annual review of cell and developmental biology. 2011; **27**: 409-440.
27. Nilsson, J., von Euler, A.M., and Dalsgaard, C.J., Stimulation of connective tissue cell growth by substance P and substance K. Nature. 1985; **315**(6014): 61-63.
28. Nye, H.L., Cameron, J.A., Chernoff, E.A., and Stocum, D.L., Regeneration of the urodele limb: a review. *Dev Dyn*. 2003; **226**(2): 280-294.
29. Oscar E. Schotté, E.G.B., Phases in Regeneration of the Urodele Limb and Their Dependence Upon te Nervous System. *Journal of Experimental Zoology*. 1944; **97**(2): 95-121.
30. Roy, S., and Gatien, S., Regeneration in axolotls: a model to aim for *Experimental gerontology*. 2008; **43**(11): 968-973.
31. Roy, S., and Levesque, M., Limb regeneration in axolotl: is it superhealing? The Scientific World Journal. 2006; **6** Suppl 112-25.
32. Satoh, A., Bryant, S.V., and Gardiner, D.M.. Nerve signaling regulates basal keratinocyte proliferation in the blastema apical epithelial cap in the axolotl (Ambystoma mexicanum). *Developmental biology*. 2012; **366**(2): 374-381.
33. Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R., Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. Science (New York, NY. 2000; **287**(5455): 1037-1040.
34. Sharma, K.K., and Niazi, I.A., Restoration of limb regeneration ability in frog tadpoles by electrical stimulation. *Indian journal of experimental biology*. 1990; **28**(8): 733-738.
35. Shi, R., and Borgens, R.B., Three-dimensional gradients of voltage during development of the nervous system as invisible coordinates for the establishment of embryonic pattern. *Dev Dyn*. 1995; **202**(2): 101-114.
36. Singer, M., The influence of the nerve in regeneration of the amphibian extremity. *The Quarterly review of biology*. 1952; **27**(2): 169-200.
37. Singer, M., The acetylcholine content of the normal forelimb regenerate of the adult newt, Triturus. *Developmental biology*. 1959; **1**(6): 603-620.
38. Singer, M., and Craven, L., The growth and morphogenesis of the regenerating forelimb of adult Triturus following denervation at various stages of development. *The Journal of experimental zoology*. 1948; **108**(2): 279-308.
39. Singer, M., Davis, M.H., and Scheuing, M.R., The Influence of Atropine and Other Neuropharmacological Substances on Regeneration of the Forelimb oin the Adule Urodele, Triturus. *Journal of Experimental Zoology*. 1960a; **143**(1): 33-45.
40. Singer, M., DAVIS, M.H., ARKOWITZ, E. S., Acetylcholinesterase Activity in the Regenerating Forelimb of the Adult Newt, Triturus. *Journal of Embryology and Experimental Morpholgy*. 1960b; **8**part298-111.
41. Singer, M., and Mutterperl, E., Nerve fiber requirements for regeneration in forelimb transplants of the newt Triturus. *Developmental biology*. 1963; **7**: 180-191.
42. Smith, S.D., Induction of partial limb regeneration in Rana pipiens by galvanic stimulation. *The Anatomical record*. 1967; **158**(1): 89-97.
43. Smith, S.D., The role of electrode position in the electrical induction of limb regeneration in subadult rats. *Bioelectrochemistry and Bioenergetics*. 1981; **8**(6): 661-670.
44. Song, F., Li, B., and Stocum, D.L., Amphibians as research models for regenerative medicine. *Organogenesis*. 2010; **6**(3): 141-150.
45. Stocum, D.L, Amphibian regeneration and stem

cells. *Current topics in microbiology and immunology.* 2004; 2801-70.
46. Stocum, D.L., The role of peripheral nerves in urodele limb regeneration. *The European journal of neuroscience.* 2011; **34**(6): 908-916.
47. Tamura, K., Ohgo, S., and Yokoyama, H., Limb blastema cell: a stem cell for morphological regeneration. Development, growth & differentiation. 2010; **52**(1): 89-99.
48. Todd, J.T., On the Process of Reproduction of the Members of the Aquatic Salamander. In, 1823; 84-96.
49. Tweedell, K.S., The urodele limb regeneration blastema: the cell potential. *The Scientific World Journal*. 2010; **10**: 954-971.
50. Wang, L., Marchionni, M.A., and Tassava, R.A., Cloning and neuronal expression of a type III newt neuregulin and rescue of denervated, nerve-dependent newt limb blastemas by rhGGF2. *Journal of neurobiology*. 2000; **43**(2): 150-158.
51. Zhao, M., Song, B., Pu, J., Wada, T., Reid, B., Tai, G., Wang, F., Guo, A., Walczysko, P., Gu, Y., *et al.,* Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. *Nature*. 2006; **442**(7101): 457-460.