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 Gatten, 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*

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 (Brookes, 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).
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).

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

Possible role of neurotransmitter 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; Jady 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).

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 midway 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 transfected with AChE. Their 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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