α7-nicotinic acetylcholine receptor signaling in cardiac regeneration

Senior Honor Thesis

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ABSTRACT

Heart disease is the leading cause of death in the U.S. Loss of heart regenerative capacity in adult mammals is a principle reason why adults fail to recover from heart diseases. In contrast, neonatal mammals possess extraordinary heart regenerative capacity. Unraveling mechanisms of heart regeneration during the neonatal period is fundamental to understanding why adult hearts fail to regenerate. Previous studies revealed that cholinergic nerves and macrophages are crucial to heart regeneration. However, their interaction during heart inflammation and regeneration is poorly investigated. My study aims to understand how α7-nicotinic acetylcholine receptor (α7-nAChR) signaling between cholinergic nerves and macrophages regulates inflammation and regeneration after heart injury. Immunohistochemistry and trichrome staining showed decreased number of proliferating cardiomyocytes and increased scar formation following myocardial infarction in neonatal α7-nAChR knockout mice. Subsequent experiments will study the activation of different macrophage lineages in neonates by flow cytometry. The study of mechanisms of heart regeneration will bring us closer to the goal of reactivating the adult human heart for cardiac repair.

INTRODUCTION

Heart failure is associated with significant mortality and healthcare expenditures around the globe. Approximately 6.5 million American adults had heart failure, costing the U.S an estimated $30 billion each year[15]. Limited ability to regenerate cardiomyocytes after heart injury is a cause of heart failure. Compared with neonatal mammals which can fully regenerate the heart within the first week of life, heart regenerative capacity in adult mammals is minimal[1]. While adult mammalian heart regeneration has not been achieved, an understanding of how the cardiomyocytes lose regenerative capacity is critical to know how to trigger regeneration in the adult heart. In turn, this knowledge will help treat heart diseases that cannot currently be treated. My study aims to unravel a potential role of acetylcholine (ACh) receptor signaling during heart regeneration in mice.

Heart regeneration is a highly coordinated process consisting of a prolonged inflammatory response, cardiomyocyte proliferation and angiogenesis. Newly formed cardiomyocytes are derived from pre-existing cardiomyocytes[10]. A recent review highlights that cholinergic nerves coordinate inflammation and reparation after heart injury in newborn mammals[2]. The inflammatory response mediated by cholinergic nerves is indispensable for cardiomyocyte proliferation and angiogenesis[2]. Pharmacological or mechanical denervation of cholinergic nerves resulted in blunted inflammatory and regenerative response in mice[3]. Cholinergic nerves are also found to impact macrophages. By binding to α7 nicotinic acetylcholine receptor (α7-nAChR) on macrophages, ACh influences the profile of cytokines secreted by macrophages[2]. Stimulating α7-nAChR downregulates macrophage secretion of pro-inflammatory cytokines including TNF-α, IFN-γ, IL-1β and IL-6[3,8]; electrical stimulation of the vagus nerve, which controls the heart, lungs and digestive system via ACh signaling, inhibits synthesis of TNF-α by macrophages[14]. In contrast, denervating the vagus nerve increases pro-inflammatory cytokines production after heart injury, leading to more fibrosis and less regeneration of the heart[9]. In short, disrupting cholinergic nerve signaling leads to excessive release of cytokines by macrophages and failure to regenerate the heart.

Despite the evidence that cholinergic nerves guide mammalian heart inflammation and regeneration, and influence macrophages via α7-nAChR, no study has explored the connection between

heart regeneration and α7-nAChR signaling in mice. Therefore, I plan to use α7-nAChR knockout (*Chrna7*$^{-/-}$) mice, with immunohistochemistry and trichrome staining to investigate how nAChR signaling influences cardiomyocyte proliferation, after heart injury. The second stage of this project involves characterizing macrophage activity during heart regeneration.

Aurora et al.[3] observed that various lineages of macrophages with distinct surface markers and transcriptional profiles were involved in heart reparation in neonatal period. They mediated disparate inflammatory responses and shaped heart regeneration after injury[3,4]. For example, macrophages can activate stromal cells, initiate fibrosis and scar formation after heart injury[17]. A common classification system divides these macrophage lineages into two groups: embryonic-derived tissue-resident macrophages (EM) and monocyte-derived blood-circulating macrophages (BM)[5,6]. EM persist throughout the life, capable of alleviating inflammation and facilitating tissue regeneration, while BM promote inflammation and fibrosis and disrupt wound healing in both neonatal and adult mice[4]. A reason of differential response of neonatal and adult heart to injury is that after injury, the neonatal heart supports the expansion of EM, which is anti-inflammatory and C-C chemokine receptor type 2 negative (CCR2-), while the adult heart recruits pro-inflammatory CCR2+ BM that replace EM[4]. Hence, in my study of α7-nAChR signaling, the ultimate goal is to characterize both EM and BM based on surface marker in order to understand how neonatal mice recruit macrophages and elicit inflammatory and regenerative responses.

Denervation of cholinergic nerves stimulates pro-inflammatory cytokines and decreases regenerative capacity[9]; therefore, I hypothesize that after myocardial infarction (MI), *Chrna7*$^{-/-}$ mice neonatal mice have lower number of proliferating cardiomyocytes, increased fibrosis, and less EM in the heart. I hypothesize that nAChR signaling primarily influences EM during the neonatal period, thereby mediating anti-inflammatory response and regeneration of injured heart.

Coronary artery ligation was performed on P1 mice to create MI. At P7, the end of the regenerative window in mice, I examined if disrupted α7-nAChR signaling in *Chrna7*$^{-/-}$ mice inhibits cardiomyocyte proliferation. At P21, I measured the size of scar formation. I used immunohistochemistry to quantify proliferative cardiomyocyte and trichrome staining to measure fibrosis area. In the next stage of this

project, we will use flow cytometry to compare the number, surface markers and lineages of macrophages in wildtype and mutant mice at different ages. After characterizing macrophage lineages, we can study expression level of genes involved in polarization and activation of macrophages.

METHODS

Establish MI mouse models

All experiments were conducted in accordance to the guidance approved by University of Wisconsin-Madison IACUC. B6.129S7-*Chrna7*$^{-/-}$ mice were purchased from The Jackson Laboratory. Both male and female mice were used since prior studies found that neonatal heart regeneration is not influenced by sex. To mimic human ischemic heart diseases, MI that permanently ligates the left anterior descending coronary artery was performed in one-day-old neonatal mice (P1) as described by Mahmoud, et al.[9]. Sham surgeries were performed as the control.

Histology

Mice were sacrificed on P7 and P21. Fixation and sectioning of heart tissues was performed as described[9] to generate paraffin-embedded heart transverse sections below the ligature in a two-chamber view. Paraffin sections were deparaffinized in xylene and rehydrated by alcohol before staining. Masson's trichrome staining were performed as described[9,13] to examine scar formation at P21. To analyze size of cardiomyocytes and the number of proliferating cardiomyocytes, immunostaining using Alexa Fluor 488 conjugate of wheat germ agglutinin (WGA), anti- phospho-histone H3 (pH3) antibodies and anti-troponin T antibodies was performed as described by Lavine et al.[4] with minor modification. Size of cardiomyocytes was calculated based on three heart and three different sections per heart. A total of 50-100 cells were measured for each section. About five sections per heart were used to quantify proliferating cardiomyocytes. Images were analyzed using ImageJ.

Statistics

Data are presented as mean±standard deviation. Statistical significance was determined by Student's Two Sample T test. Differences were considered statistically significant when $P \leqslant 0.05$.

RESULT

Size of cardiomyocytes increased in Chrna7$^{-/-}$ mice

At P21 after MI, a slight increase in heart weight in *Chrna7*$^{-/-}$ mice was observed compared to wildtype MI mice. However, the increase was not statistically significant. WGA staining against glycoproteins on cell membrane outlined the contour of cardiomyocytes. Based on colocalization with nucleus and troponin staining, area of cardiomyocytes on the cross section was quantified. The average size of P21 cardiomyocytes in *Chrna7*$^{-/-}$ mice was larger than wildtype mice (n=3 for wildtype and *Chrna7*$^{-/-}$, $p \leqslant 0.0001$).

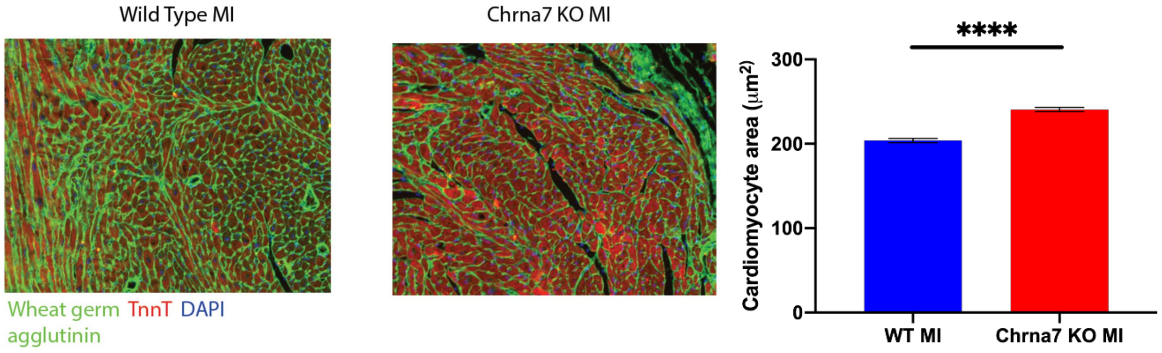

Figure 1. Quantification of the size of ventricular cardiomyocytes in P21 mice. WGA staining outlined boundary of cell membrane in green. Average size of cardiomyocytes in *Chrna7*$^{-/-}$ mice was larger than wildtype mice (n=3 for wildtype and *Chrna7*$^{-/-}$, $p \leqslant 0.0001$). Area of 50-100 cells per heart section was measured.

Deletion of Chrna7 impaired cardiomyocyte proliferation after MI

Immunohistochemistry using antibodies against pH3 captured cardiomyocytes that were going through G2 and M phase of mitosis and cytokinesis. Colocalization with DAPI and troponin T staining enabled identification of proliferating cardiomyocytes. The average number of proliferating

cardiomyocytes was similar across Sham mice and *Chrna7*$^{-/-}$ MI mice, but much lower than wildtype MI mice across the entire heart section (n ⩾5 for wildtype, n=6 for *Chrna7*$^{-/-}$ MI, p⩽0.0001). *Chrna7*$^{-/-}$ resulted in 65% decrease of the number of proliferating cardiomyocytes after MI in P7 mice. The disruption of α7-nAChR signaling significantly impaired the regenerative response of cardiomyocytes.

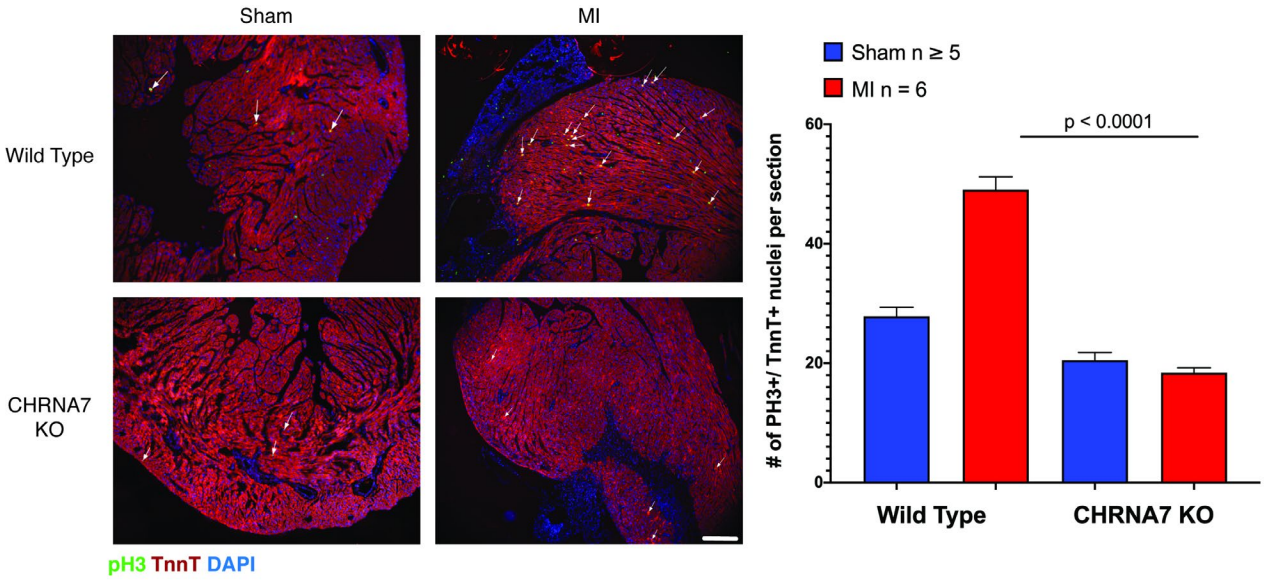

Figure 2. Quantification of the number of proliferating ventricular cardiomyocyte per heart at P7. Immunohistochemistry showed average number of proliferating cardiomyocytes per heart was higher in wildtype than in *Chrna7*$^{-/-}$ P7 MI mice (n ⩾5 for wildtype, n=6 for *Chrna7*$^{-/-}$ MI, p⩽0.0001). Arrow indicates proliferating cardiomyocytes. Scale bar sets to 100 μm.

Deletion of Chrna7 increased scar formation

Scar formation as a result of collagen deposition is an indicator of incomplete cardiac regeneration. By measuring the size of fibrosis tissue stained in blue by trichrome staining, area of scar in heart sections was quantified. The average scar size of *Chrna7*$^{-/-}$ mice was about twice as large as that in wildtype mice (n=6 for wildtype MI, n=4 for *Chrna7*$^{-/-}$ MI, p0.01). P21 *Chrna7*$^{-/-}$ had more fibrosis from ligation site to apex, showing excess deposition of collagen and glycosaminoglycans and reduced capacity to regenerate functional cardiomyocytes.

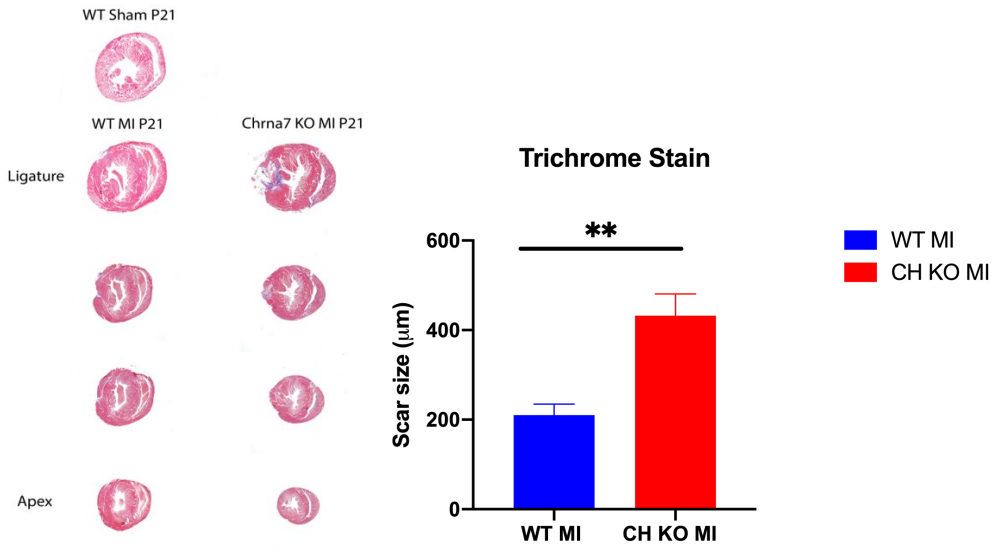

Figure 3. Measurement of ventricle scar size in MI mice at P21. Representative images showed ventricular sections from ligation site to apex in trichrome staining for fibrosis (blue) and cardiomyocytes (pink). Average fibrosis area per heart was two times larger in *Chrna7*$^{-/-}$ than in wildtype P21 mice (n=6 for wildtype MI, n=4 for *Chrna7*$^{-/-}$ MI, $p \leqslant 0.01$).

DISCUSSION

This study found that deleting *Chrna7* resulted in increased cardiomyocytes size and fibrosis after MI in neonatal mice. *Chrna7* knockout also decreased the number of proliferating cardiomyocytes after MI in neonatal mice. IHC and trichrome staining demonstrated that architecture of cardiomyocytes was not greatly affected by *Chrna7* deletion. The functionality and electrophysiology of the heart in mutant mice, however was not studied. In summary, regenerative capacity of neonatal heart was impaired by disruption of α7-nAChR signaling.

WGA staining revealed larger size of cardiomyocytes in *Chrna7*$^{-/-}$ mice with and without MI. While disrupting α7-nAChR signaling induced abnormal growth of cardiomyocytes during heart development, the difference of size of the entire heart was not significantly different (data not shown). Since the orientation of cardiomyocytes in the slides could not be determined, whether the growth was concentric or eccentric and the underlying mechanisms of the growth was unknown. Since cardiomyocytes had minimal expression of α7-nAChR, the growth may be triggered by signaling from other cells in the

heart or physiological adaptation to high blood pressure. However, the cause of the growth hence remains to be explored.

The response to MI begins with immune cell infiltration, inflammation and tissue digestion that lasts approximately 4 days in mice. After the initial phase, inflammatory signaling decreases and cellular proliferation is trigger, which promotes both scar formation and cardiomyocyte proliferation[18]. Collagen deposition start within hours of tissue injury[18]. As in Figure 3, disruption of α7-nAChR signaling increased fibrosis near ligation site. Two fold increases of fibrosis area indicated impaired repair response to MI injury. The heart of *Chrna7*$^{-/-}$ mice failed to restore normal tissue architecture under the blockage of α7-nAChR signaling. Cardiomyocyte contration is impaired by stiff scar tissue. The presence of large scar tissue will ultimately lead to cardiac dysfunction and chronic heart failure. Therefore, the ideal treatment of heart failure is both to activate cardiomyocyte proliferation and reduce scar formation.

Since macrophages orchestra fibrosis, local macrophages subsets may be regulated differently in *Chrna7*$^{-/-}$ mice, as indicated by additional deposition of collagen and extracellular matrix in injuried site, excessive proliferation of stromal cells and impaired ability to produce growth factor and clear cell debris. The composition of macrophages in injured neonatal hearts is found to be different from that of adult heart in mice[4]. Inflammation, fibrosis and tissue regeneration induced by macrophages are not mutually exclusive, and macrophage lineages can differentiate into distinct subtyes based on local signals[17]. Because nAch signaling plays an important role in stimulating macrophage subsets, it is important to elucidate what macrophages subsets and how they are activated by nerve signaling in neontal heart after MI. We will continue on optimization of FACS protocol in order to quantify cardiac macrophages subtypes. RNA-Seq assessments of the immune populations in the heart may be necessary to grasp the whole picture of the change brought by cholinergic inflammatomry pathway. Transcriptional, epigenetic study and identification of surface marker of macrophages may also be performed as part of future study.

The results confirmed that loss of the α7-nAChR signaling impaired cardiac regeneration. However, since *Chrna7* is highly expressed in multiple types of neurons and fibroblast in mice[16], full body knockout of the α7-nAChR may affect other signaling pathways involved in heart regeneration. Hence we

should analyze expression level of *Chrna7* in various cell types in wildtype mice. In future study we also use Cre-recombinase to create a macrophage conditional knockout of α7-nAChR.

This study can shed light on potential therapeutic interventions targeting α7-nAChR to activate the non-neuronal cholinergic anti-inflammatory pathway and restore regenerative capacity in adult mammals. This study will lay a foundation for improving the outcome of surgical repair for congenital heart diseases during the neonatal period. Future research can explore the mechanisms of the non-neuronal cholinergic system in heart regeneration by focusing on interaction with immune cells and signaling factors secreted by neurons, cardiac fibroblasts and cardiomyocytes that have immune-regulatory functions[11]. Because the majority of EM are replaced by BM in an adult heart after injury[4], mechanisms and means to maintain EM and restrict BM infiltration are also worth investigating.

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REFERENCES

[1] Porrello, E. et al., 2011., Transient regenerative potential of the neonatal mouse heart. *Science*, 331(6020), pp.1078–1080.
[2] Tracey, K., 2009. Reflex control of immunity. *Nature Reviews Immunology*, 9(6), 418-41828.
[3] Aurora, A.B. et al., 2014. Macrophages are required for neonatal heart regeneration. *The Journal of clinical investigation*, 124(3), pp.1382–92.
[4] Kory J. Lavine et al., 2014. Distinct macrophage lineages contribute to disparate patterns of cardiac recovery and remodeling in the neonatal and adult heart. *Proceedings of the National Academy of Sciences*, 111(45), pp.16029–34.
[5] Epelman, S., Liu, P., & Mann, D., 2015. Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nature Reviews Immunology*, 15(2), pp.91–104.
[6] Hashimoto, D. et al., 2013. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*, 38(4), pp.792–804.
[7] Morimoto, H. et al., 2006. Cardiac overexpression of monocyte chemoattractant protein-1 in transgenic mice prevents cardiac dysfunction and remodeling after myocardial infarction. *Circulation*, 114(18), p.262.
[8] Kawashima et al., 2015. Non-neuronal cholinergic system in regulation of immune function with a focus on α7 nAChRs. *International Immunopharmacology*, 29(1), pp.127–134.
[9] Mahmoud, A., et al., 2015. Nerves regulate cardiomyocyte proliferation and heart regeneration. *Developmental Cell*, 34(4), pp.387–99.
[10] Porrello, E., Mahmoud, A., et al., 2013. *PNAS*. 110 (1) 187-192
[11] Lambiase, Alessandro et al., 1997. Human CD4+ T cell clones produce and release nerve growth factor and express high-affinity nerve growth factor receptors. *The Journal of Allergy and Clinical Immunology*, 100(3), pp.408–414.

[12] Rauch et al., 2012. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *The Journal of Experimental Medicine*, 209(1), pp.123–137.
[13] Mahmoud, A., et al., 2014. Surgical models for cardiac regeneration in neonatal mice. *Nature Protocols*, 9(2), pp.305–311.
[14] Wang, H. et al., 2002. Nicotinic acetylcholine receptor α7 subunit is an essential regulator of inflammation. *Nature*, 421(6921), pp.384–388.
[15] Benjamin E., et al., 2019. Heart disease and stroke statistics—2019 update: a report from the American Heart Association. *Circulation*.139;56–e528.
[16] Expression Altas. Expression of Chrna7 in mouse. EMBL-EBI, 2020. https://www.ebi.ac.uk/gxa/genes/ensmusg00000030525?bs=%7B%22mus%20musculus%22%3A%5B%22CELL_TYPE%22%5D%7D&ds=%7B%22kingdom%22%3A%5B%22animals%22%5D%7D#baseline
[17] Wynn, T. and Vannella, K., 2016. Macrophages in Tissue Repair, Regeneration, and Fibrosis. *Immunity*, 44.
[18] Prabhu SD, Frangogiannis NG., 2016. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ Res.*119(1):91-112. Epub 2016/06/25.