Developmental Biology 358 (2011) 309–317

Contents lists available at SciVerse ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

In vivo calcium dynamics during neural crest cell migration and patterning using GCaMP3

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ARTICLE INFO

Article history:
Received for publication 3 June 2011
Revised 6 August 2011
Accepted 7 August 2011
Available online 16 August 2011

Keywords:
Chick
Neural crest
Cell migration
Calcium transients
GCaMP3
Time-lapse imaging
Confocal
Sympathetic ganglia

ABSTRACT

Examining calcium dynamics within the neural crest (NC) has the potential to shed light on mechanisms that regulate complex cell migration and patterning events during embryogenesis. Unfortunately, typical calcium indicators are added to culture media or have low signal to noise after microinjection into tissue that severely limit analyses to cultured cells or superficial events. Here, we studied in vivo calcium dynamics during NC cell migration and patterning, using a genetically encoded calcium sensor, GCaMP3. We discovered that trunk NC cells displayed significantly more spontaneous calcium transients than cranial NC cells, and during cell aggregation versus cell migration events. Spontaneous calcium transients were more prevalent during NC cell aggregation into discrete sympathetic ganglia (SG). Blocking of N-cadherin activity in trunk NC cells near the presumptive SG led to a dramatic decrease in the frequency of spontaneous calcium transients. Detailed analysis and mathematical modeling of cell behaviors during SG formation showed NC cells aggregated into clusters after displaying a spontaneous calcium transient. This approach highlights the novel application of a genetically encoded calcium indicator to study subsets of cells during ventral events in embryogenesis.

Introduction

During embryogenesis, beautiful discrete structures arise from complex cell behaviors that include long distance cell migration and cell aggregation. Complex events such as these require rapid signaling mechanisms to regulate the timing of cells to move and coalesce such that discrete structures develop at precise locations. Calcium transients or spontaneous increases of intracellular calcium represent one example of rapid signaling within cells. Calcium transients have been observed in complex embryonic events such as convergent extension (Wallingford et al., 2001) and early neuronal differentiation and patterning (Ciccolini et al., 2003; Gu and Spitzer, 1995; Spitzer, 2006). Advances in live imaging are providing a means to better visualize and quantitate cell movements within embryos (Bower et al., 2011; Supatto et al., 2009). However, two of the remaining roadblocks to in vivo calcium imaging are the lack of fluorescence indicators that are easily deliverable into embryos and a means to quantitatively correlate calcium transients with specific cell behaviors and morphogenetic events.

Visualization of calcium transients in embryos has been challenging due to the limited number of available fluorescence indicators and methods of delivery (Hires et al., 2008). Typical calcium indicators, such as Fluo-4 AM (Heidenreich et al., 2008) require invasive delivery into embryos or can be soaked into cells and tissue in vitro by external application. This severely limits the ability to accurately deliver calcium indicators to discrete cell subpopulations within the developing embryo during precise times of complex morphogenetic events. Genetically encoded calcium indicators have emerged as an exciting set of tools to overcome this roadblock. Specifically, GCaMP3, has been developed and applied to monitor complex cell behavioral events in several adult model systems (Tian et al., 2009; Xiang et al., 2010). Additionally, GCamp3 is non-toxic to embryos and can be transfected into cells or delivered by electroporation to specific subpopulations of cells. Thus, genetically encoded calcium indicators provide a means to study calcium transients in vivo in a targeted manner and have the potential to advance our knowledge of complex cell behavioral events during embryogenesis.

One of the prominent examples in embryogenesis where long distance cell migration and cell aggregation give rise to discrete structures is the formation of the peripheral nervous system (PNS) (Kulesa and Gammill, 2010; Kulesa et al., 2009). During formation of the PNS, trunk neural crest (NC) cells travel ventral migratory pathways and aggregate into discrete cell clusters of the dorsal root (DRG) and sympathetic ganglia (SG) in a repeating pattern along the vertebrate axis (Gammill et al., 2006; Kasemeier-Kulesa et al., 2005, 2006). One of the major questions of PNS development is how trunk NC cell behaviors are regulated in space and time to produce the pattern of the DRG and SG. Surprisingly, trunk NC cell behaviors are

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0012-1606/$ – see front matter © 2011 Published by Elsevier Inc.
doi:10.1016/j.ydbio.2011.08.004

more complex than originally thought. The formation of the primary SG occurs after NC cells travel to the dorsal aorta (DA) in a discrete migratory stream, then disperse along the dorsal aorta before re-aggregating into a discrete cluster (Kasemeier-Kulesa et al., 2005). Thus, it is clear that insights into calcium dynamics could shed light on the cell migration and patterning events during PNS development.

In this study, we examined the calcium dynamics during chick NC cell migration using GCaMP3 and confocal time-lapse imaging. The GCaMP3 vector was electroporated into premigratory NC cells and calcium transients were visualized in vivo in whole chick embryos and in trunk sagittal slice explants. We found differences in the spatiotemporal pattern of fluorescence activity of GCaMP3-labeled cranial and trunk NC cells. We characterized the differences in NC cell calcium transient dynamics, especially during phases when NC cells stopped and coalesced into discrete structures of the SG. We analyzed changes in calcium transients after blocking N-cadherin activity in trunk NC cells during SG formation. Lastly, we constructed a mathematical model, based on detailed NC cell behavioral measurements, that quantitatively correlated calcium transients with NC cell aggregation into discrete SG. Our approach provides a powerful tool for analyzing in vivo NC cell calcium dynamics during cell migratory and patterning events in embryogenesis.

Experimental procedures

Embryo labeling

Fertilized white leghorn chicken eggs (Phil's Fresh Eggs, Forreston, IL) were incubated at 38 °C in a humidified incubator until the appropriate stages. Eggs were rinsed with 70% ethanol and 3 mL of albumin was removed before windowing the eggshell and staging (Hamburger and Hamilton, 1951). A solution of 10% India ink (Pelikan Fount; www.mrart.com, Houston, TX) in Howard Ringer's solution was injected below the area opaca to visualize each embryo. Embryos were injected and electroporated between 6 and 8 somites for cranial labeling; and 9 and 11 somites for trunk NC cell labeling (Kasemeier-Kulesa et al., 2006; Stark and Kulesa, 2007) with a cocktail of DNA plasmids appropriate for each experiment to fluorescently label premigratory NC cells located in the dorsal trunk neural tube. Eggs were resealed with adhesive tape and incubated at 38 °C until the appropriate stage. After incubation, we evaluated each embryo prior to manipulation for brightness and uniformity of GCaMP3 labeling using a FITC filter and selected embryos that were developing normally.

GCaMP3 under the control of a CAG promoter was a kind gift from L. Looger. Either a monomeric red fluorescent protein mCherry (gift of R. Lansford) was co-injected for a cytoplasmic label, or to aid in cell tracking, we co-injected a nuclear localized H2B-mCherry (gift from R. Krumlauf lab) both under a CMV promoter. All constructs were used at initial concentration of 5ug/ul.

Embryo mounting for imaging

For cranial studies, whole embryos were harvested at HH Stage 11 and mounted dorsal side up on a Millicell culture insert (Millipore, Billerica, MA) inside a glass bottom culture dish (Mattek Corp, Ashland, MA) with approximately 1.5 mL Neurobasal media (Invitrogen, Carlsbad, CA) supplemented with B27, L-glutamine and Pen/Strep.

For trunk imaging, embryos were allowed to incubate 2–3 days post electroporation then selected for imaging at HH Stages 17–20. Sagittal slice explants were selected at the brightest axial level, and between the forelimb and hindlimb level to consistently acquire data on trunk NC cell migration and patterning. Tissue was prepared and mounted as in (Kasemeier-Kulesa et al., 2007) and allowed 5 min of rest at 37 °C before imaging. Briefly, approximately 6–8 somite long sections of the trunk were excised down the neural tube midline and gently placed on a Millicell culture insert in a Mattek dish with Neurobasal media. The culture dish was then sealed with parafilm to prevent evaporation. For N-cadherin antibody experiments, the antibody (AbCam, ab11340, Cambridge, MA) was diluted to 300 ug/mL in a solution of Howard Ringer's and a very small amount of Fast Green FCF (Sigma-Aldrich, St. Louis, MO) was added to visualize injection into the tissue. We microinjected into the mounted tissue sample lateral to NC cells dispersed along the anterior–posterior axis adjacent to the dorsal aorta, similar to (Kasemeier-Kulesa et al., 2006), and all along the tissue length instead of just one somite length. The culture chamber was sealed and time-lapse imaging was performed immediately after injection.

For in vitro studies, embryos were electroporated between 6 and 8 somites and allowed to re-incubate for approximately 2 h. Well-labeled and healthy embryos were harvested and the cranial (mid-rhombomere (r3–r5)) region of the neural tube was excised and mounted on a fibronectin and poly-L lysine coated Mattek dish (McKinney et al., 2011). The explanted cells were incubated for 12 h to allow the cells to move away from the neural tube before imaging.

Time-lapse imaging

An LSM5 Pascal or LSM-510 (Zeiss, Thornwood, NY) were used to collect single plane images in 2 channels (GCaMP3 and mCherry) of the labeled NC cells every 30 s. The confocal pinhole was adjusted to 2.5 Airy units to collect more fluorescence in the single plane image so that frequent images could be collected without damaging the tissue with as much laser light exposure as in a z-stack. A chamber was constructed around the microscope with a heater to sustain 38 °C while imaging (Kasemeier-Kulesa et al., 2006). The GCaMP3 was excited with a 488nm laser line and imaged with a 505–530 nm bandpass filter and the mCherry was excited using 543 nm laser light and collected with a 560–615 nm bandpass filter. Either a 10×/0.45 NA Plan-Apochromat (Zeiss) or a 10×/0.5 NA Fluar (Zeiss) objective was used. All time-lapse imaging sessions were performed for at least 4 h and up to 10 h.

Data analysis

Images were collected in AIM software (Zeiss) and processed in Imaris (Bitplane, South Windsor, CT). Calcium transients were located in Imaris by hand with the "spots" function and statistical information was exported to analysis software in Matlab (Mathworks Inc., Natick, MA). The spot size in Imaris was created so that the measurements did not extend out of the cell body to contain background fluorescence or in the case of H2B-mCherry labeling, only include the nuclear area. $\Delta F/F_0$ was calculated for each calcium transient by $(I_t–I_0)/I_0$ where I_t was the mean intensity of the cell during a calcium transient within the spot created in Imaris and I_0 was the mean intensity in the frame preceding the calcium transient. This value was calculated for both GCaMP3 and mCherry channels. To eliminate the possibility of changes in GCaMP3 intensity due to cell movement rather than calcium concentration changes, cells with increases $\Delta F/F_0$ in the green channel that were accompanied by increases $\Delta F/F_0$ in the red channel were excluded from analysis. Boundaries of the tissue regions were determined from the confocal images and used to automatically assign every calcium transient to a presumptive tissue position.

Mathematical model and simulations

Cells with calcium transients were examined in Imaris before and after each calcium transient. Only cells near and in the SG region were examined for directional movement. The average direction of cell motion before and after each calcium transient was determined by hand in general terms as being either toward or away from the center