

# Catenin signaling controls phrenic motor neuron development and function during a narrow temporal window

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## Abstract

Phrenic Motor Column (PMC) neurons are a specialized subset of motor neurons (MNs) that provide the only motor innervation to the diaphragm muscle and are therefore essential for survival. Despite their critical role, the mechanisms that control phrenic MN development and function are not well understood. Here, we show that catenin-mediated cadherin adhesive function is required for multiple aspects of phrenic MN development. Deletion of  $\beta$ - and  $\gamma$ -*catenin* from MN progenitors results in perinatal lethality and a severe reduction in phrenic MN bursting activity. In the absence of catenin signaling, phrenic MN topography is eroded, MN clustering is lost and phrenic axons and dendrites fail to grow appropriately. Despite the essential requirement for catenins in early phrenic MN development, they appear to be dispensable for phrenic MN maintenance, as catenin deletion from postmitotic MNs does not impact phrenic MN topography or function. Our data reveal a fundamental role for catenins in PMC development and suggest that distinct mechanisms are likely to control PMC maintenance.

## 29 Introduction

30 Breathing is an essential motor behavior that is required for survival. In mammals,  
31 contraction of the diaphragm muscle is critical for bringing oxygenated air into the lungs during  
32 inspiration (Greer, 2012). Diaphragm contractions are mediated by a specialized subset of  
33 motor neurons (MNs), Phrenic Motor Column (PMC) neurons that reside in the cervical spinal  
34 cord and project their axons along the phrenic nerve in the thoracic cavity to reach the  
35 diaphragm. Phrenic MNs exhibit distinct properties from other MN subtypes, including tight  
36 clustering and stereotyped axonal and dendritic morphologies. While they are derived from a  
37 common MN progenitor domain, phrenic MNs acquire their unique features through the activity  
38 of a selective transcriptional program that distinguishes them from other MNs (Chaimowicz et  
39 al., 2019; Machado et al., 2014; Philippidou et al., 2012; Vagnozzi et al., 2020). Phrenic-specific  
40 transcription factors (TFs) initiate and maintain the expression of a distinct set of genes,  
41 including a unique combination of cell surface adhesion molecules (Machado et al., 2014;  
42 Vagnozzi et al., 2020). While many of these molecular markers show specific and sustained  
43 expression in phrenic MNs, their functions in phrenic MN development and maintenance have  
44 not been tested.

45 We previously identified a distinct combinatorial cadherin code that defines phrenic MNs,  
46 which includes both the broadly expressed type I N-cadherin and a subset of specific type II  
47 cadherins (Vagnozzi et al., 2020). Cadherins are calcium-dependent transmembrane cell  
48 adhesion molecules that interact with cytosolic catenin proteins to induce changes in the actin  
49 cytoskeleton, thus regulating many neuronal processes such as migration, topography and  
50 morphology (Seong et al., 2015). For example, cadherins regulate cortical neuron migration  
51 (Martinez-Garay, 2020), hippocampal dendritic growth and branching (Bekirov et al., 2008; Esch  
52 et al., 2000; Yu and Malenka, 2003), as well as dendrite morphogenesis and arborization within  
53 the visual and olfactory systems (Duan et al., 2018; Hirano and Takeichi, 2012; Masai et al.,  
54 2003; Riehl et al., 1996; Tanabe et al., 2006; Zhu and Luo, 2004). In the spinal cord, cadherins  
55 engage  $\beta$ - and  $\gamma$ -catenins to establish the segregation and settling position of MN cell bodies  
56 (Demireva et al., 2011; Dewitz et al., 2019; Dewitz et al., 2018; Price et al., 2002).  $\beta$ -catenin is  
57 required in muscle for neuromuscular junction (NMJ) formation and function, however it appears  
58 to act redundantly with  $\gamma$ -catenin in MNs, as only joint  $\beta$ - and  $\gamma$ -catenin inactivation leads to  
59 disorganization of MN subtypes, including PMC neurons (Demireva et al., 2011; Li et al., 2008;  
60 Vagnozzi et al., 2020). However, it is unknown whether  $\beta$ - and  $\gamma$ -catenins have additional roles  
61 in phrenic MN development and function, and whether they continue to be required after initial  
62 PMC topography has been established.

63 Here, we show that catenin activity is required for proper respiratory behavior and robust  
64 respiratory output. After MN-specific deletion of  $\beta$ - and  $\gamma$ -catenin, mice display severe  
65 respiratory insufficiency, gasp for breath, and die within hours of birth. Using phrenic nerve  
66 recordings, we determined that catenins are crucial for respiratory motor output, as MN-specific  
67 catenin inactivation leads to a striking decrease in phrenic MN activity. We further show that  
68 catenins are required to establish phrenic MN cell body settling position, as well as PMC axonal  
69 and dendritic morphology. Finally, we show that catenins are only required for PMC  
70 development and function during a narrow developmental window, as catenin deletion from  
71 postmitotic MNs does not impact respiratory output. Our data demonstrate a fundamental role  
72 for the cadherin-catenin cell adhesion complex in phrenic MN development and respiratory  
73 function and indicate that distinct pathways likely act to maintain PMC function.

## 74 **Materials and Methods**

### 75 **Mouse genetics**

76 The *β-catenin* (Brault et al., 2001) and *γ-catenin* (Demireva et al., 2011) alleles,  
77 *Olig2::Cre* (Dessaud et al., 2007) and *ChAT::Cre* (Lowell et al., 2006) lines were generated as  
78 previously described and maintained on a mixed background. Mouse colony maintenance and  
79 handling was performed in compliance with protocols approved by the Institutional Animal Care  
80 Use Committee of Case Western Reserve University. Mice were housed in a 12-hour light/dark  
81 cycle in cages containing no more than five animals at a time.

### 82 **Immunohistochemistry and in situ hybridization**

83 Immunohistochemistry was performed as previously described (Philippidou et al., 2012;  
84 Vagnozzi et al., 2020), on tissue fixed for 2 hours in 4% paraformaldehyde (PFA) and  
85 cryosectioned at 16μm. Wholemounts of diaphragm muscles were stained as described  
86 (Philippidou et al., 2012). The following antibodies were used: goat anti-Scip (1:5000; Santa  
87 Cruz Biotechnology, RRID:AB\_2268536), mouse anti-Islet1/2 (1:1000, DSHB,  
88 RRID:AB\_2314683) (Tsuchida et al., 1994), rabbit anti-neurofilament (1:1000; Synaptic  
89 Systems, RRID:AB\_887743), rabbit anti-synaptophysin (1:250, Thermo Fisher,  
90 RRID:AB\_10983675), and α-bungarotoxin Alexa Fluor 555 conjugate (1:1000; Invitrogen,  
91 RRID:AB\_2617152). Images were obtained with a Zeiss LSM 800 confocal microscope and  
92 analyzed with Zen Blue, ImageJ (Fiji), and Imaris (Bitplane). Phrenic MN number was quantified  
93 using the Imaris “spots” function to detect cell bodies that coexpressed high levels of Scip and  
94 Isl1/2 in a region of interest limited to the left and right sides of the ventral spinal cord.

### 95 **Dil tracing**

96 For labeling of phrenic MNs, crystals of carbocyanine dye, Dil (Invitrogen, #D3911) were  
97 pressed onto the phrenic nerves of eviscerated embryos at e18.5, and the embryos were  
98 incubated in 4% PFA at 37°C in the dark for 4-5 weeks. Spinal cords were then dissected,  
99 embedded in 4% low melting point agarose (Invitrogen) and sectioned using a Leica VT1000S  
100 vibratome at 100 to 150μm.

### 101 **Positional analysis**

102 MN positional analysis was performed as previously described (Dewitz et al., 2019;  
103 Dewitz et al., 2018). MN positions were acquired using the “spots” function of the imaging  
104 software Imaris (Bitplane) to assign x and y coordinates. Coordinates were expressed relative to  
105 the midpoint of the spinal cord midline, defined as position x=0, y=0. To account for  
106 experimental variations in spinal cord size, orientation, and shape, sections were normalized to  
107 a standardized spinal cord whose dimensions were empirically calculated at e13.5 (midline to  
108 the lateral edge=390μm). We analyzed every other section containing the entire PMC (20-30  
109 sections in total per embryo).

### 110 **Dendritic orientation analysis**

111 For the analysis of dendritic orientation, we superimposed a radial grid divided into  
112 eighths (45 degrees per octant) centered over phrenic MN cell bodies spanning the entire length  
113 of the dendrites. We drew a circle around the cell bodies and deleted the fluorescence  
114 associated with them. Fiji (ImageJ) was used to calculate the fluorescent intensity (IntDen) in  
115 each octant which was divided by the sum of the total fluorescent intensity to calculate the  
116 percentage of dendritic intensity in each area.

### 117 **Electrophysiology**

118 Electrophysiology was performed as previously described (Vagnozzi et al., 2020). Mice  
119 were cryoanesthetized and rapid dissection was carried out in 22-26°C oxygenated Ringer's  
120 solution. The solution was composed of 128mM NaCl, 4mM KCl, 21mM NaHCO<sub>3</sub>, 0.5mM  
121 NaH<sub>2</sub>PO<sub>4</sub>, 2mM CaCl<sub>2</sub>, 1mM MgCl<sub>2</sub>, and 30mM D-glucose and was equilibrated by bubbling in  
122 95% O<sub>2</sub>/5% CO<sub>2</sub>. The hindbrain and spinal cord were exposed by ventral laminectomy, and  
123 phrenic nerves exposed and dissected free of connective tissue. A transection at the  
124 pontomedullary boundary rostral to the anterior inferior cerebellar artery was used to initiate  
125 fictive inspiration. Electrophysiology was performed under continuous perfusion of oxygenated  
126 Ringer's solution from rostral to caudal. Suction electrodes were attached to phrenic nerves just  
127 proximal to their arrival at the diaphragm. The signal was band-pass filtered from 10Hz to 3kHz  
128 using AM-Systems amplifiers (Model 3000), amplified 5,000-fold, and sampled at a rate of  
129 50kHz with a Digidata 1440A (Molecular Devices). Data were recorded using AxoScope  
130 software (Molecular Devices) and analyzed in Spike2 (Cambridge Electronic Design). Burst  
131 duration and burst activity were computed from 4-5 bursts per mouse, while burst frequency  
132 was determined from 10 or more minutes of recording time per mouse. Burst activity was  
133 computed by rectifying and integrating the traces with an integration time equal to 2 seconds,  
134 long enough to encompass the entire burst. The maximum amplitude of the rectified and  
135 integrated signal was then measured and reported as the total burst activity.

### 136 **Plethysmography**

137 Conscious, unrestrained P0 mice were placed in a whole body, flow-through  
138 plethysmograph (emka) attached to a differential pressure transducer (emka). We modified  
139 10ml syringes to use as chambers, as smaller chambers increase signal detection in younger  
140 mice. Experiments were done in room air (79% nitrogen, 21% oxygen). Mice were placed in the  
141 chamber for 30 seconds at a time, for a total of three to five times, and breathing parameters  
142 were recorded. Mice were directly observed to identify resting breaths. At least ten resting  
143 breaths were analyzed from every mouse. Data are presented as fold control, where the control  
144 is the average of 2 littermates in normal air.

### 145 **Experimental design and statistical analysis**

146 For all experiments a minimum of three embryos per genotype, both male and female,  
147 were used for all reported results unless otherwise stated. The Shapiro-Wilk test was used to  
148 determine normality. All data, with the exception of electrophysiology burst activity data in  
149 figures 2 and 6, showed normal distribution and p-values were calculated using unpaired, two-  
150 tailed Student's *t* test. Burst activity data in figures 2 and 6 showed non-normal distribution and  
151 p-values were calculated using the Mann-Whitney test.  $p < 0.05$  was considered to be  
152 statistically significant, where \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , and \*\*\*\*  $p < 0.0001$ . Data are  
153 presented as box and whisker plots with each dot representing data from one mouse unless  
154 otherwise stated. Small open squares in box and whisker plots represent the mean.

## 155 **Results**

### 156 **Catenin signaling is required for survival, proper respiratory behavior and phrenic MN** 157 **activation**

158 Phrenic MNs express a distinct combinatorial cadherin code (Machado et al., 2014;  
159 Vagnozzi et al., 2020), but the collective contribution of these molecules to phrenic MN  
160 development, maintenance and function has not been established. We previously found that  
161 phrenic MNs express the type I *N-cadherin* (*N-cad*) and the type II cadherins *Cdh6*, *9*, *10*, *11*,  
162 and *22* (Vagnozzi et al., 2020). To investigate the role of cadherin signaling in phrenic MN

163 development, we eliminated cadherin signaling in MN progenitors by inactivating  $\beta$ - and  $\gamma$ -  
164 *catenin* using a *Olig2::Cre* promoter ( $\beta$ -*catenin* *flox/flox*;  $\gamma$ -*catenin* *flox/flox*; *Olig2::Cre*, referred  
165 to as  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice).  $\beta$ - and  $\gamma$ -catenin are obligate intracellular factors required for cadherin-  
166 mediated cell adhesive function and catenin deletion enables us to interrogate the full repertoire  
167 of cadherin actions in phrenic MNs (Figure 1a). We find that  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice are born alive but  
168 die within 24 hours of birth and often display severe flexion of the wrist joint (Figure 1b).

169 In order to assess breathing in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice, we utilized unrestrained whole body flow-  
170 through plethysmography at postnatal day (P)0 (Figure 1c). We found that  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice have  
171 a 45% reduction in tidal volume (the amount of air inhaled during a normal breath), while  
172 respiratory frequency is not affected (Figure 1d-e). This results in an average 45% reduction in  
173 overall air drawn into the lungs per minute (minute ventilation, figure 1e), indicating  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>   
174 mice likely die from respiratory failure. Our findings indicate that catenin signaling is necessary  
175 for proper respiratory behavior and survival. To further examine respiratory circuitry intrinsic to  
176 the brainstem and spinal cord, we performed suction recordings of the phrenic nerve in isolated  
177 brainstem-spinal cord preparations (Figure 2a). These preparations display fictive inspiration  
178 after the removal of inhibitory networks in the pons via transection, and thus represent a robust  
179 model to interrogate circuit level changes. We examined whether catenin deletion impacts  
180 circuit output at embryonic day (e)18.5/P0, shortly before  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice die. We observed a  
181 striking reduction in the activation of phrenic MNs in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice (Figure 2b). While bursts in  
182 control mice exhibit large peak amplitude, bursts in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice were either of very low  
183 amplitude (~85%) or non-detectable (~15%). After rectifying and integrating the traces, we  
184 found a nearly 70% decrease in total burst activity in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice (Figure 2c). Our data  
185 indicate that cadherin signaling is imperative for robust activation of phrenic MNs during  
186 inspiration.

### 187 **Catenins establish phrenic MN topography and organization**

188 What accounts for the loss of phrenic MN activity in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice? We asked whether  
189 early phrenic MN specification, migration and survival are impacted after catenin inactivation.  
190 We acquired transverse spinal cord sections through the entire PMC at e13.5 and stained for  
191 the phrenic-specific TF Scip and the MN-specific TF Isl1/2, to label all phrenic MNs. We found a  
192 cluster of Scip+ MNs in the ventral cervical spinal cord of both control and  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice,  
193 indicating that early phrenic MN specification is unperturbed (Figure 3a). However, we observed  
194 a significant reduction in phrenic MN numbers in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice, indicating that catenin  
195 signaling is necessary for phrenic MN survival (Figure 3b).

196 While phrenic MNs are normally distributed along the rostrocaudal axis, we found that  
197 they sometimes show migratory defects, where several phrenic MNs remain close to the midline  
198 instead of fully migrating (Figure 3c, arrow), and also appear to shift both ventrally and medially  
199 in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice. To quantitate PMC cell body position, each phrenic MN was assigned a  
200 cartesian coordinate, with the midpoint of the spinal cord midline defined as (0,0).  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>   
201 mice displayed a significant shift in phrenic MN cell body position, with cell bodies shifting  
202 ventrally towards the edge of the spinal cord and towards the midline (Figure 3c-f). We  
203 quantified the average ventrodorsal and mediolateral phrenic MN position per embryo and found  
204 a significant change in phrenic MN position in both axes in  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice (Figure 3g).  
205 Correlation analysis indicated that control and  $\beta\gamma$ -*cat*<sup>MN $\Delta$</sup>  mice are dissimilar from each other  
206 ( $r=0.47$ , Figure 3h), indicating that catenins establish phrenic MN coordinates during  
207 development.



208 In addition to changes in cell body position, we also noticed that phrenic MNs appear to  
209 lose their tight clustering in  $\beta\gamma\text{-cat}^{MN\Delta}$  mice. PMC clustering is thought to be critical for the proper  
210 development of the respiratory system because it facilitates recruitment of motor units through  
211 electrical coupling in the embryo to compensate for weak inspiratory drive (Greer and Funk,  
212 2005). In order to determine PMC clustering defects, we used Imaris to measure the average  
213 distance between phrenic MNs. We found that  $\beta\gamma\text{-cat}^{MN\Delta}$  mice had a nearly 50% increase in the  
214 average distance between neighboring cells (Figure 3i), indicating that the cadherin/catenin  
215 adhesive complex contributes to the formation of a tightly clustered phrenic motor column.

### 216 **Catenins are required for phrenic MN dendrite and axon growth**

217 Since cadherin/catenin signaling is imperative for phrenic MN organization, we also  
218 asked whether any other aspects of phrenic MN development, such as dendritic and axon  
219 growth, might rely on catenin actions. We examined dendritic orientation in control and  $\beta\gamma\text{-cat}^{MN\Delta}$   
220 mice by injecting the lipophilic dye dil into the phrenic nerve at e18.5 (Figure 4a). Dil  
221 diffuses along the phrenic nerve to label both PMC cell bodies and dendrites. Consistent with  
222 our earlier observations, we found that phrenic cell bodies are often scattered in  $\beta\gamma\text{-cat}^{MN\Delta}$  mice.  
223 Interestingly, even phrenic MNs that are significantly displaced correctly project along the  
224 phrenic nerve (arrows, figure 4a), indicating that changes in cell body position do not impact  
225 axon trajectory choice. In control mice, phrenic MN dendrites branch out in dorsolateral to  
226 ventromedial directions; in  $\beta\gamma\text{-cat}^{MN\Delta}$  mice, however, they exhibit stunted growth, defasciculation  
227 and a failure to extend in the dorsolateral direction (Figure 4a).

228 To quantify these changes, we superimposed a radial grid divided into octants onto the  
229 dendrites and measured the fluorescent intensity in each octant after removal of any  
230 fluorescence associated with the cell bodies. Zero degrees was defined by a line running  
231 perpendicular from the midline through the center of cell bodies. In control mice, the majority of  
232 dendrites project in the dorsolateral direction (0-90 degrees), representing 40-45% of the overall  
233 dendritic intensity (Figure 4b, d, e). Ventrally projecting dendrites (180-225 degrees and 315-  
234 360 degrees) were also prominent, giving rise to nearly 30% of the overall dendritic intensity  
235 (Figure 4b, d, e). We found that catenin deletion resulted in a striking reduction in dorsolateral  
236 dendrites, together with a significant increase in ventral dendrites (Figure 4c-e), indicating that  
237 cadherins are necessary for establishing phrenic MN dendritic orientation. These changes in  
238 phrenic dendritic topography in  $\beta\gamma\text{-cat}^{MN\Delta}$  mice may impact their targeting by respiratory  
239 populations in the brainstem, leading to the reduction in phrenic MN activation observed.

240 We then asked whether catenins might also play an analogous role in phrenic axon  
241 extension and arborization. We examined diaphragm innervation in control and  $\beta\gamma\text{-cat}^{MN\Delta}$  mice  
242 at e18.5. We found that  $\beta\gamma\text{-cat}^{MN\Delta}$  mice display a lack of innervation in the ventral diaphragm  
243 (Figure 5a, arrow), while the parts of the diaphragm that are innervated show a reduction in  
244 terminal arborization complexity (Figure 5a, star). Quantitation of total phrenic projections  
245 revealed a significant reduction in overall diaphragm innervation (Figure 5b-c). Collectively our  
246 data point to a catenin requirement for phrenic MN topography and axon and dendrite  
247 arborization, suggesting that these changes in early phrenic MN development lead to loss of  
248 phrenic MN activity and perinatal lethality due to respiratory insufficiency.

### 249 **A narrow temporal requirement for catenin signaling in phrenic MN topography and** 250 **function.**

251 Given the essential role for catenins in early phrenic MN development, we wanted to  
252 further understand the temporal dynamics of cadherin signaling, and asked whether sustained

253 catenin expression is required for maintenance of the respiratory circuit. We used a *ChAT::Cre*  
254 promoter to specifically delete  $\beta$ - and  $\gamma$ -catenin in postmitotic MNs ( $\beta$ -catenin *flox/flox*;  $\gamma$ -catenin  
255 *flox/flox*; *ChAT::Cre*, referred to as  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>  mice).  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>  mice survive to adulthood  
256 and do not display respiratory insufficiency or gasping at birth. We first assessed cell body  
257 position and found no changes between control and  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>  mice (Figure 6a). Injecting dil  
258 into the phrenic nerve also revealed no differences in dendritic orientation between control and  
259  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>  mice (Figure 6b). To assess respiratory circuit function, we performed phrenic  
260 nerve recordings in isolated brainstem-spinal cord preparations in control and  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>   
261 mice at P4, and found that  $\beta\gamma$ -cat<sup>ChATMN $\Delta$</sup>  mice exhibit similar phrenic MN bursting frequency,  
262 burst duration and overall activity as control mice (Figure 6c-d). Our data suggests that  
263 cadherins engage catenin signaling during a short temporal window early in development to  
264 shape respiratory motor output, but appear to be dispensable once early phrenic MN  
265 topography and morphology have been established.

266

## 267 Discussion

268 Phrenic MNs are a critical neuronal population that is essential for breathing, yet the  
269 molecular mechanisms that control their development and maintenance have remained elusive.  
270 Here, we show that catenin-mediated cadherin adhesive function is required for phrenic MN  
271 organization, axonal and dendritic arborization, and respiratory output during a narrow  
272 developmental window. While catenins have a critical role in early phrenic MN development,  
273 they appear to be dispensable for maintaining the morphology and function of these MNs. Our  
274 findings indicate that distinct molecular pathways are likely to mediate the establishment and  
275 maintenance of respiratory motor circuits at different timepoints throughout development and  
276 adulthood.

277

278 Catenins appear to be critical for phrenic MN organization. We find that catenin  
279 inactivation leads to both ventral shifts in PMC position and loss of clustering between cell  
280 bodies. While we observe similar shifts in cell body position when we inactivate 4 out of the 6  
281 cadherins expressed in PMC neurons (cadherins N, 6, 9 and 10-*N<sup>MN $\Delta$</sup> 6910<sup>-/-</sup>* mice), we do not  
282 see a loss of clustering in these mice (Vagnozzi, 2022). This could suggest that retaining  
283 expression of the remaining PMC-specific cadherins, 11 and 22, is sufficient to maintain the  
284 phrenic MN distinct tight clustering organization. Alternatively, our data could indicate that cell  
285 non-autonomous cadherin function plays a predominant role in MN clustering, and that  
286 eliminating cadherin signaling from all MNs leads to scattering and mixing of MN populations,  
287 causing disorganization not seen when solely eliminating PMC-specific cadherins. Despite  
288 differentially affecting PMC clustering, we observe similar changes in phrenic MN activity in  
289 *N<sup>MN $\Delta$</sup> 6910<sup>-/-</sup>* and  $\beta\gamma$ -cat<sup>MN $\Delta$</sup>  mice, suggesting that MN clustering may not significantly contribute to  
290 phrenic MN activity. Alternatively, since the loss of activity we observe in both mouse models is  
291 so severe, it may mask a subtler impact of clustering to PMC activity patterning and  
292 synchronization. Decoupling PMC clustering from changes in neuronal morphology and cell  
293 adhesion loss will help distinguish the contribution of each of these properties to respiratory  
294 motor output.

295

296 Our data show that cadherin signaling is required both for the elaboration of PMC axons  
297 and dendrites, however the impact of catenin inactivation on dendrites appears to be more  
298 severe. Phrenic MNs are able to elaborate axons in  $\beta\gamma$ -cat<sup>MN $\Delta$</sup>  mice and axonal topography and

299 orientation are mostly preserved, with some minor loss of terminal arborization. Dendrites  
300 however appear to be severely stunted, project haphazardly and their topography is lost. This  
301 indicates that cadherins have a much more predominant role in dendritic rather than axonal  
302 elaboration. While many signaling pathways contribute to phrenic axon growth and diaphragm  
303 innervation, including HGF/MET (Sefton et al., 2022), Slit/Robo (Charoy et al., 2017) and  
304 Col25a1 (Tanaka et al., 2014), to our knowledge, cadherins are the first cell adhesion molecules  
305 to be implicated in phrenic MN dendritic development.

306  
307       Loss of catenin-mediated cadherin adhesive function results in a dramatic reduction of  
308 phrenic MN activity that leads to perinatal lethality. This could be due to the loss of descending  
309 inputs from brainstem respiratory centers that provide excitatory drive to initiate diaphragm  
310 contraction during inhalation. The dramatic change in PMC dendritic coordinates is likely to  
311 contribute significantly to the loss of presynaptic inputs and respiratory activity. Dendrites  
312 represent the largest surface area of neurons, and thus receive the majority of synaptic input. In  
313 sensory-motor circuits, proprioceptive inputs are primarily located on the dendrites of motor  
314 neurons, and different motor pools exhibit distinct, stereotyped patterns of dendritic arborization  
315 that contribute to sensory-motor specific connectivity (Balaskas et al., 2019; Vrieseling and  
316 Arber, 2006). This mode of cadherin action in respiratory circuits would be consistent with  
317 cadherin-dependent targeting mechanisms in the retina, where combinatorial codes of cadherin  
318 expression serve to direct axons and dendrites of synaptically connected neurons to their  
319 correct laminar targets (Duan et al., 2014; Duan et al., 2018; Osterhout et al., 2011).

320  
321       In addition to establishing phrenic MN dendritic morphology, cadherins could directly  
322 contribute to phrenic connectivity through establishing a molecular recognition program between  
323 phrenic MN dendrites and pre-motor axons. Due to their restricted and selective expression in  
324 neural populations, cadherins are thought to function in circuit assembly by dictating synaptic  
325 specificity. Cadherin expression often reflects the functional connections formed in a circuit,  
326 suggesting they may represent a molecular code dictating the formation of selective synaptic  
327 connections (Suzuki et al., 1997). Cadherins are expressed on dendrites, axons, and growth  
328 cones of developing neurons (Basu et al., 2015) and have been visualized at synapses in both  
329 pre and postsynaptic compartments (Bartelt-Kirbach et al., 2010; Benson and Tanaka, 1998;  
330 Bozdagi et al., 2000; Fannon and Colman, 1996; Manabe et al., 2000; Suzuki et al., 2007;  
331 Uchida et al., 1996; Yamagata et al., 1995). Therefore, cadherins could function to establish  
332 respiratory neuron connectivity independently of their role in dictating axonal and dendritic  
333 targeting, at the level of the synapse, as it has been described in the hippocampus (Basu et al.,  
334 2017; Williams et al., 2011). Future experiments will determine the primary mode of cadherin  
335 action in respiratory circuit formation.

336  
337       While early cadherin inactivation in MN progenitors results in dramatic changes in  
338 phrenic MN morphology and activity and leads to perinatal death, cadherin inactivation in  
339 postmitotic MNs does not impact respiratory output. This result provides initial evidence  
340 supporting a predominant role for cadherins in shaping dendritic orientation, as they appear to  
341 be dispensable once phrenic MN morphology has been established. Our findings indicate a  
342 model in which cadherins may function to direct the dendrites and axons of pre and  
343 postsynaptic neurons to the correct location, while additional cell adhesion molecules dictate  
344 synaptic connectivity. In support of this hypothesis, we have identified a number of cell adhesion  
345 molecules that are specifically expressed in phrenic MNs but are not required for their



346 morphology. Our results also indicate that distinct mechanisms may function to maintain  
347 respiratory circuit integrity after initial formation. Understanding how these critical circuits are  
348 maintained in adulthood is essential, as loss of respiratory function underlies lethality in many  
349 neurodegenerative diseases such as Amyotrophic Lateral Sclerosis (ALS).

350

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358 **References**

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- 480
- 481



## 482 Figure legends

### 483 **Figure 1. Catenin signaling is required for survival and proper respiratory behavior**

484 **a)** B- and  $\gamma$ -catenin are obligate intracellular factors required for cadherin-mediated cell  
485 adhesive function. We utilized inactivation of  $\beta$ - and  $\gamma$ -catenin in MNs ( $\beta$ -catenin flox/flox;  $\gamma$ -  
486 catenin flox/flox; *Olig2::Cre*, referred to as  $\beta\gamma$ -cat<sup>MNΔ</sup> mice) as a strategy to define the function of  
487 cadherin signaling in phrenic MNs. **b)** Appearance of P0 control and  $\beta\gamma$ -cat<sup>MNΔ</sup> mice.  $\beta\gamma$ -cat<sup>MNΔ</sup>  
488 mice are cyanotic and die shortly after birth. **c)** Experimental setup for whole body  
489 plethysmography experiments. **d)** Representative 10 second traces in room air from control and  
490  $\beta\gamma$ -cat<sup>MNΔ</sup> mice at P0. **e)**  $\beta\gamma$ -cat<sup>MNΔ</sup> mice display reduced tidal volume resulting in a 45%  
491 reduction in overall ventilation (n=4 control, n=6  $\beta\gamma$ -cat<sup>MNΔ</sup> mice).

### 492 **Figure 2. Catenin signaling controls phrenic MN activation**

493 **a)** Schematic of brainstem-spinal cord preparation, which displays fictive inspiration after  
494 removal of the pons. Suction electrode recordings were taken from the phrenic nerve in the  
495 thoracic cavity at e18.5/P0. **b)** Enlargement of single respiratory bursts reveals a reduction in  
496 burst amplitude and overall activity in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice. Partial (initial 350ms) bursts are shown.  
497 While 85% of  $\beta\gamma$ -cat<sup>MNΔ</sup> mice display respiratory bursts, 15% show no bursts throughout the  
498 recording period. **c)**  $\beta\gamma$ -cat<sup>MNΔ</sup> mice exhibit nearly 70% reduction in burst activity (n=10 control,  
499 n=10  $\beta\gamma$ -cat<sup>MNΔ</sup> mice).

### 500 **Figure 3. Catenins establish phrenic MN topography and organization**

501 **a)** Rostral to caudal distribution of e13.5 phrenic MN cell bodies (yellow, defined by the  
502 expression of Scip in green and Isl1/2 in red) in control and  $\beta\gamma$ -cat<sup>MNΔ</sup> mice. While phrenic MN  
503 cell bodies in control mice gradually shift towards more ventral positions at caudal locations,  
504 phrenic cell bodies are located ventrally in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice even at rostral levels. In addition, cell  
505 bodies in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice appear less clustered. **b)** Reduction in phrenic MN number in  $\beta\gamma$ -cat<sup>MNΔ</sup>  
506 mice. **c)** Reconstructed distribution of cell bodies in control and  $\beta\gamma$ -cat<sup>MNΔ</sup> mice. Occasional cell  
507 bodies remain near the progenitor zone in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice (arrow), while others seem to be  
508 dragged out of the spinal cord by their axon (arrowhead). **d)** Contour density plot of phrenic cell  
509 body position in control and  $\beta\gamma$ -cat<sup>MNΔ</sup> mice at e13.5. V-D  $\mu$ m; ventrodorsal position, M-L  $\mu$ m;  
510 mediolateral position. (0,0) represents the center of the spinal cord in both dimensions. **e-f)**  
511 Density plots of ventrodorsal (e) and mediolateral (f) cell body position in control and  $\beta\gamma$ -cat<sup>MNΔ</sup>  
512 mice. **g)** Quantification of ventrodorsal and mediolateral position, showing significant shifts in  
513  $\beta\gamma$ -cat<sup>MNΔ</sup> mice. **h)** Correlation analysis of phrenic MN positional coordinates in control and  $\beta\gamma$ -  
514 cat<sup>MNΔ</sup> mice. 0 is no correlation, while 1 is a perfect correlation. **i)** Phrenic MNs have an  
515 increased average distance to their neighboring phrenic MNs in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice, indicating loss  
516 of clustering. Scale bar= 25 $\mu$ m.

### 517 **Figure 4. Catenins control phrenic MN dendritic growth and orientation**

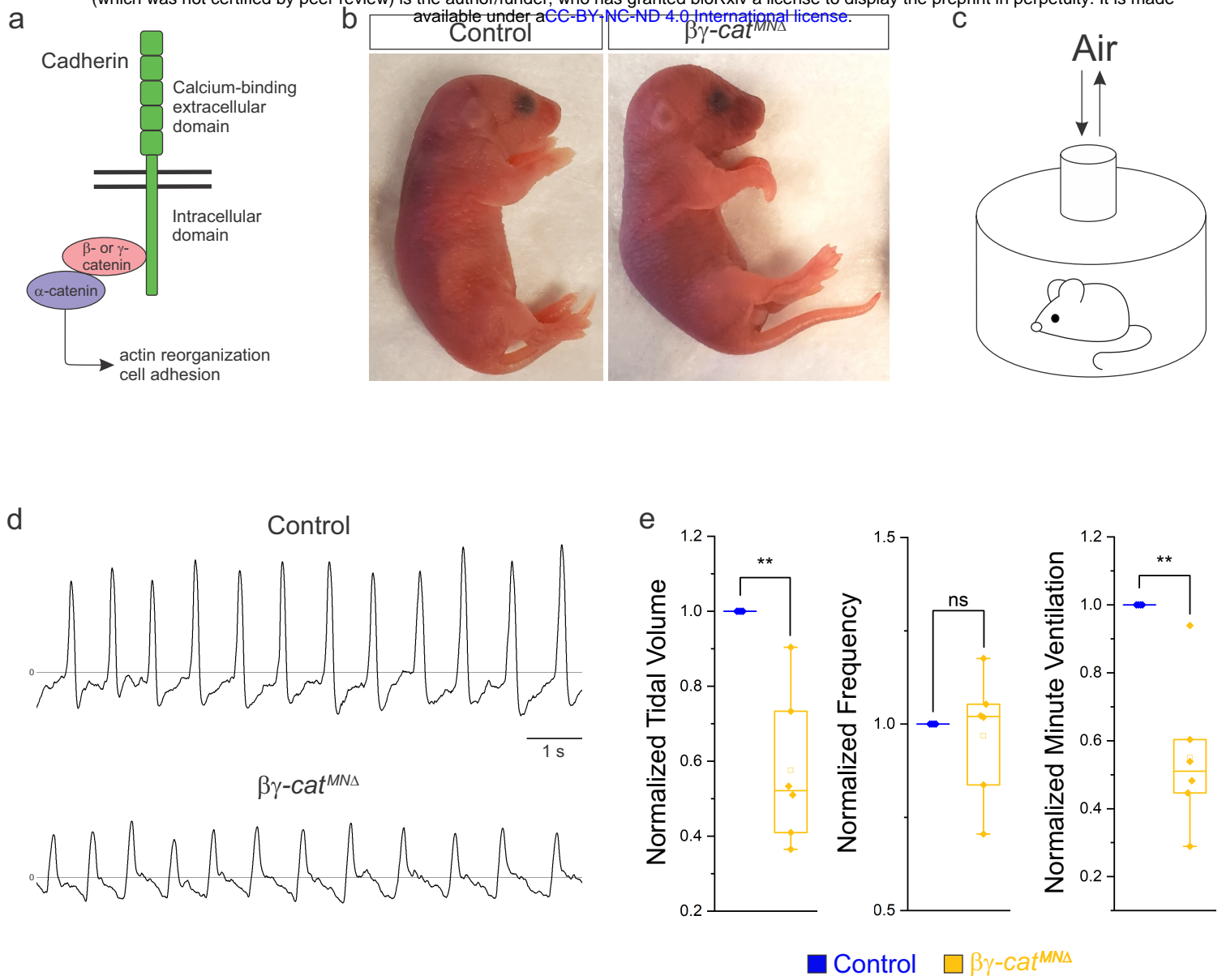
518 **a)** Rostral to caudal extent of phrenic MN dendrites, as revealed by dil injections into the phrenic  
519 nerve in control and  $\beta\gamma$ -cat<sup>MNΔ</sup> mice. Scale bar= 100 $\mu$ m. **b-d)** Radial plot of the normalized  
520 fluorescent intensity in each octant in control (b, d) and  $\beta\gamma$ -cat<sup>MNΔ</sup> (c-d) mice. Zero degrees  
521 represents a line through the center of the phrenic MN cell bodies that is perpendicular to the  
522 midline. Dendrites in  $\beta\gamma$ -cat<sup>MNΔ</sup> mice shift ventrally, with a loss of dorsolateral projections. **e)**  
523 Quantification of the proportion of dendritic fluorescent intensity from 0 to 90 degrees (left,  
524 dorsolateral) and from 180 to 225 degrees and 315 to 360 degrees (right, ventral) in control and  
525  $\beta\gamma$ -cat<sup>MNΔ</sup> mice.

526 **Figure 5. Catenins regulate phrenic MN axonal arborization**

527 **a)** Diaphragm innervation in control and  $\beta\gamma\text{-cat}^{MND}$  mice.  $\beta\gamma\text{-cat}^{MND}$  mice display a reduction in  
528 ventral diaphragm innervation (arrow) and arborization complexity (star) at e18.5. Motor axons  
529 are labeled in green (combination of neurofilament light chain/synaptophysin) and  
530 neuromuscular junctions in red ( $\alpha$ -bungarotoxin, btx). Scale bar= 500 $\mu\text{m}$ . **b)** Phrenic projections  
531 were traced and quantified in ImageJ. **c)** Quantification of diaphragm innervation in control and  
532  $\beta\gamma\text{-cat}^{MND}$  mice.

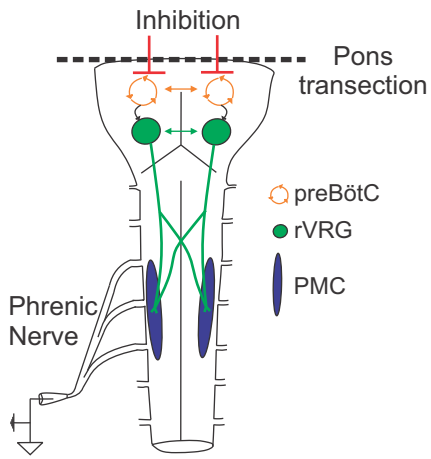
533 **Figure 6. A narrow temporal requirement for catenin signaling in phrenic MN topography**  
534 **and function.**

535 **a)** Examples of phrenic MN cell body distribution in control and  $\beta\gamma\text{-cat}^{ChATMN\Delta}$  mice at e13.5.  
536 *ChAT::Cre*-mediated catenin deletion from postmitotic MNs does not affect their position or  
537 clustering. Scale bar= 25 $\mu\text{m}$ . **b)** Dil injections into the phrenic nerve reveal similar dendritic  
538 architecture in control and  $\beta\gamma\text{-cat}^{ChATMN\Delta}$  mice at e18.5. Scale bar= 100 $\mu\text{m}$ . **c)** Representative  
539 suction electrode recordings from the phrenic nerve in P4 control and  $\beta\gamma\text{-cat}^{ChATMN\Delta}$  mice show  
540 similar levels of phrenic MN activation. **d)** Burst frequency, duration and integrated activity are  
541 unchanged in  $\beta\gamma\text{-cat}^{ChATMN\Delta}$  mice (n=7 control, n=7  $\beta\gamma\text{-cat}^{ChATMN\Delta}$  mice).

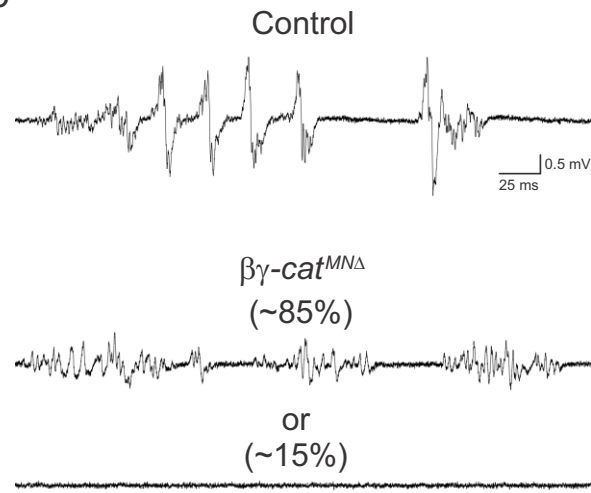


Vagnozzi\_Figure 1

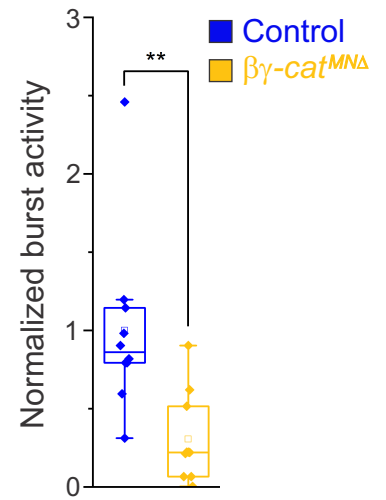
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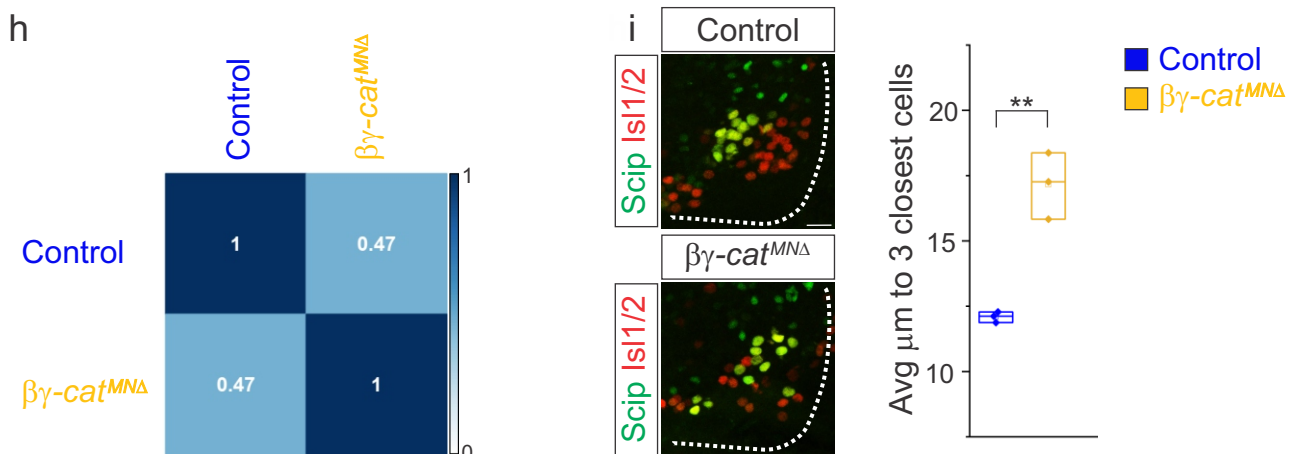
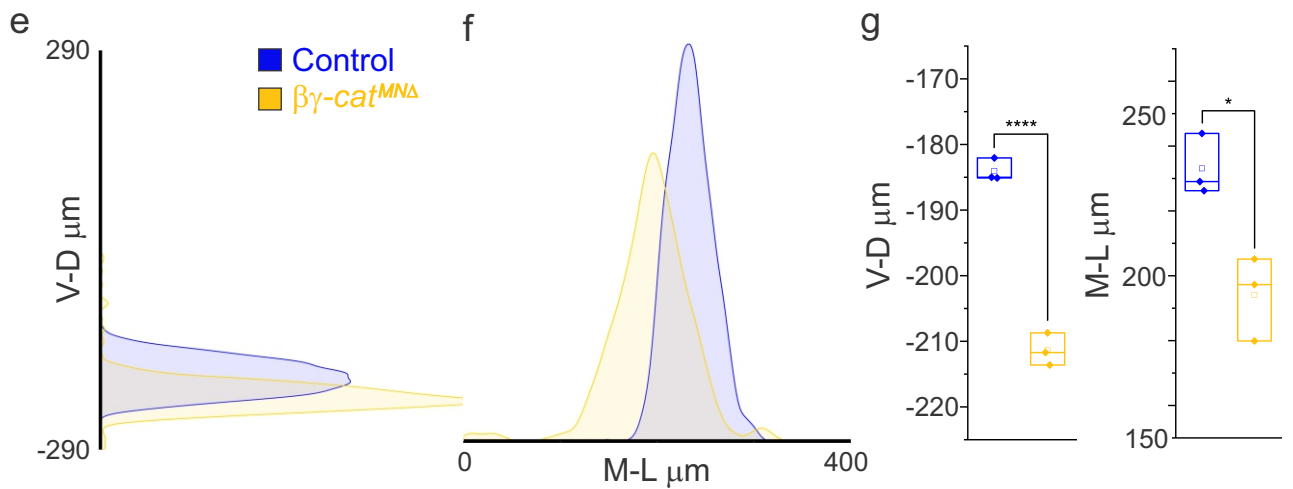
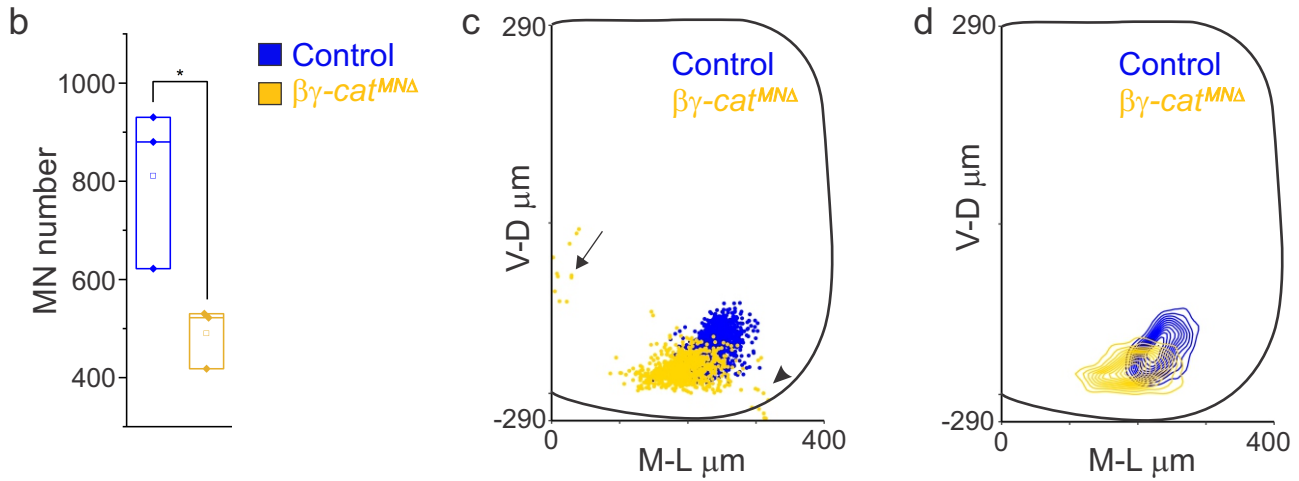
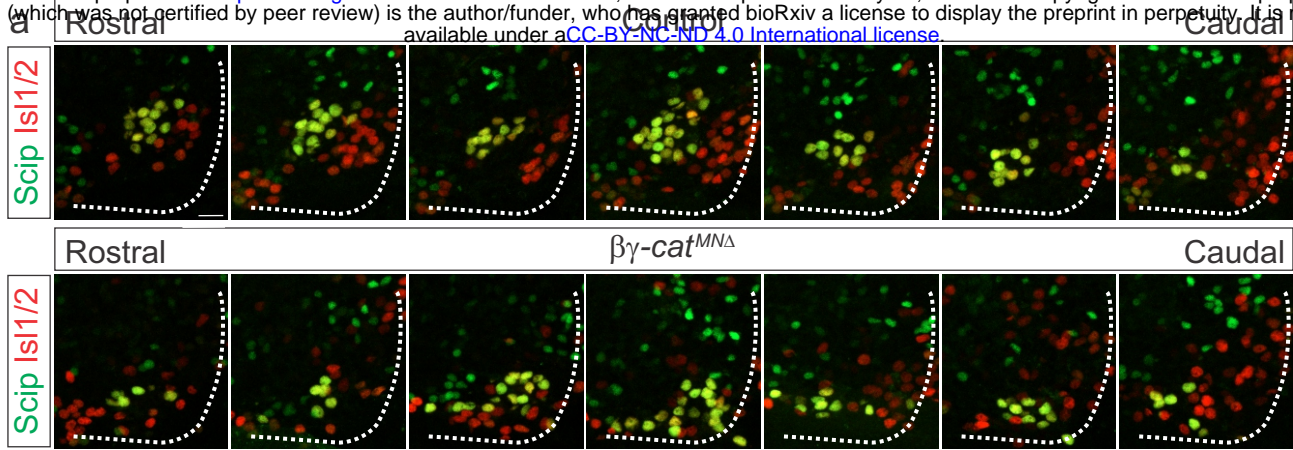
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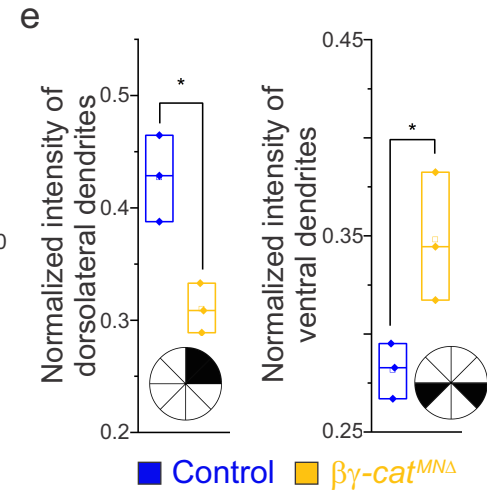
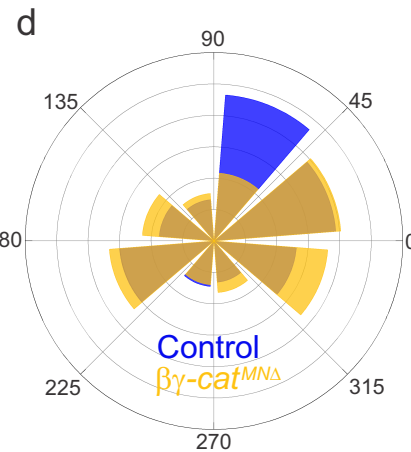
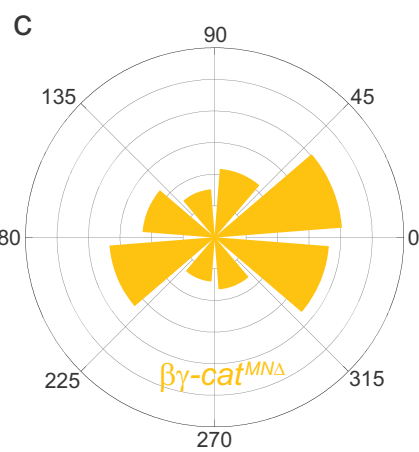
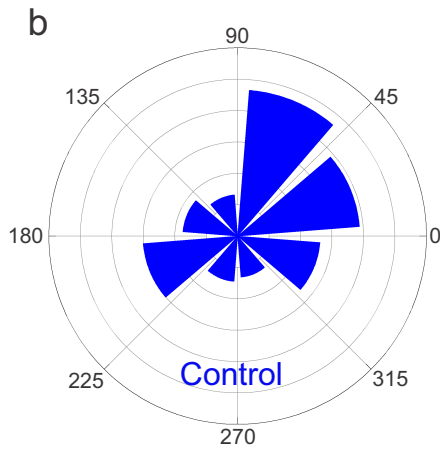
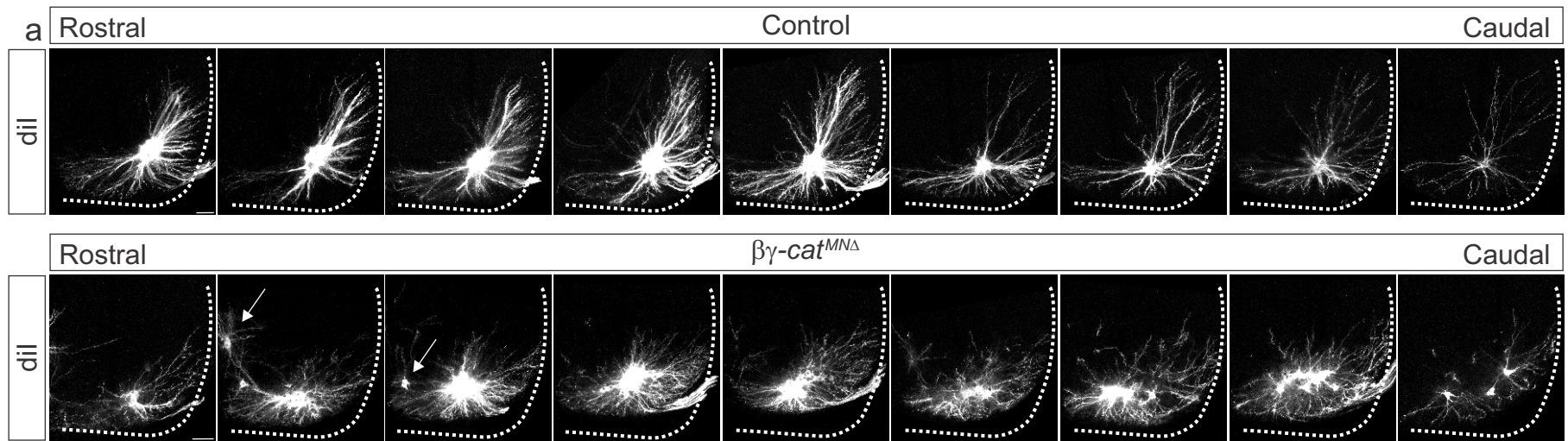
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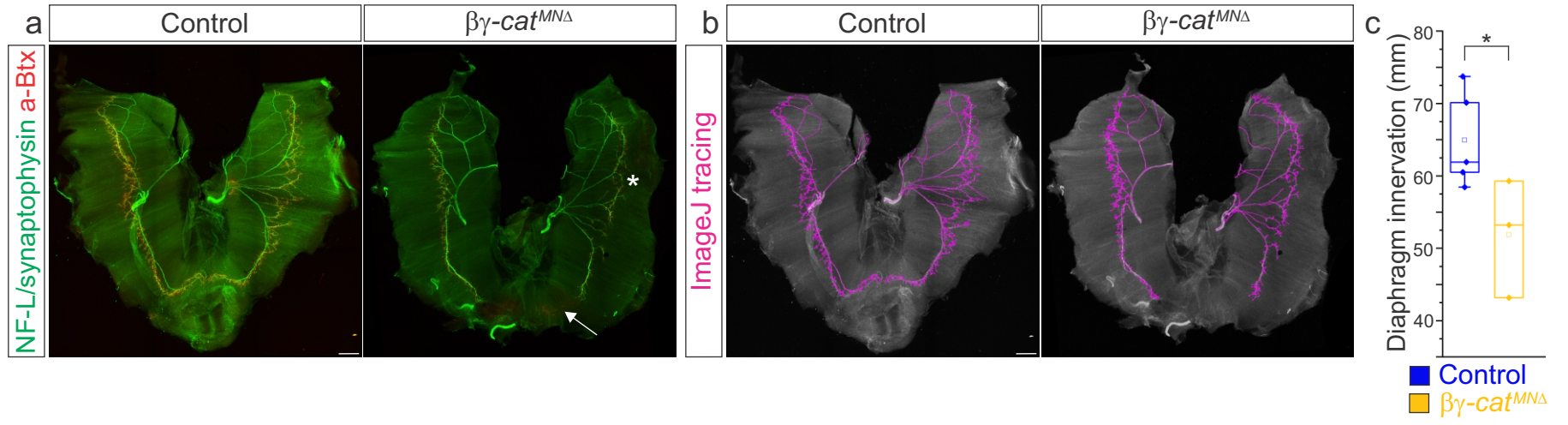


Vagnozzi\_Figure 2









Vagnozzi\_Figure 5

