

Cingulate cortex shapes early postnatal development of social vocalizations

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This **important** study investigates the influence of the cingulate cortex on the development of the social vocalizations of marmoset monkeys by making bilateral lesions of this brain area in neonatal animals. The evidence supporting the authors' claims is **convincing**. The work will be of broad interest to cognitive neuroscientists, speech and language researchers, and primate neuroscientists.

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Abstract

The social dynamics of vocal behavior has major implications for social development in humans. We asked whether early life damage to the anterior cingulate cortex (ACC), which is closely associated with socioemotional regulation more broadly, impacts the normal development of vocal expression. The common marmoset provides a unique opportunity to study the developmental trajectory of vocal behavior, and to track the consequences of early brain damage on aspects of social vocalizations. We created ACC lesions in neonatal marmosets and compared their pattern of vocalization to that of age-matched controls throughout the first 6 weeks of life. We found that while early life ACC lesions had little influence on the production of vocal calls, developmental changes to the quality of social contact calls and their associated sequential and acoustic characteristics were compromised. These animals made fewer social contact calls, and when they did, they were short, loud and monotonic. We further determined that damage to ACC in infancy results in a permanent alteration in downstream brain areas known to be involved in social vocalizations, such as the amygdala and periaqueductal gray. Namely, in the adult, these structures exhibited diminished GABA-immunoreactivity relative to control animals, likely reflecting disruption of the normal inhibitory balance following ACC deafferentation. Together, these data indicate that the normal development of social vocal behavior depends on the ACC and its interaction with other areas in the vocal network during early life.

Introduction

Vocal behavior is a critical mediator of social communication through different life stages of many animals, and particularly in social species such as primates¹. The common marmoset is a small, arboreal monkey with an elaborate repertoire of acoustic calls. While the meaning and usage of most marmoset vocalizations are not well understood, research has shown that different call types convey information about their social organization, environment, and the presence of food or predators^{2,1}. Moreover, these calls undergo developmental progression. During the first postnatal months, the acoustic properties and usage of marmoset infant vocalizations change markedly. For example, for different call types, parameters such as duration and frequency follow typical trajectories during the first months of life, transitioning from an immature babbling phase with a mixture of proto-calls to a more discrete and contingent usage of adult-like calls^{3,4}. Recent evidence also suggests that parental or social interaction plays a significant role in the proper development of normal vocal behavior, raising the prospect that important aspects of marmoset vocal behavior are learned^{5,6}. Thus, a failure to convey appropriate social information through vocal calls could potentially influence how the infant develops its social interactive skills.

In this study, we focus on the anterior cingulate cortex (ACC) and its contribution to vocal behavior and its development in early life. The ACC is a limbic cortical region known to contribute to vocal behaviors^{7,8}, and particularly those associated with emotional states^{9–11}. Electrical stimulation of the most rostral segment of the ACC elicits vocalizations^{12–14}, whereas ACC ablations limit spontaneous vocalizations¹⁵ and voluntary control of vocal behavior^{16,17}. Its dense anatomical connections with the amygdala^{18,19} underscore its role in shaping the affective component of vocalizations^{20–23}. At the same time, its descending projections to the periaqueductal gray^{24,25} endow the ACC direct control over activating the brainstem vocalization pathway^{7,26,27}.

Vocal production leads to expression of immediate early genes in the ACC²⁸, with early studies reporting that infant ACC lesions abolish the characteristic cries that infants normally issue when separated from their mother²⁹. These findings implicate the ACC in volitional and emotional control over vocal output.

Longitudinal monitoring of vocal behavior provides a tractable, high dimensional readout of the development of socio-affective circuits. It also provides a means to investigate how early life disruption to brain areas such as the ACC might affect the normal progression of social interaction. If the ACC contributes to the early-life maturation of vocal behavior, then neonatal ACC lesions should hamper the normal control of emotional vocal utterances. Here, we performed excitotoxic ACC lesions in neonatal marmosets and tracked their vocal behaviors, comparing them to age-matched controls throughout the first 6 weeks of life, and examined the impact of the early life lesion on interconnected brain regions in the vocal production network. We demonstrate that animals with neonatal damage to the ACC retained their capacity to issue calls. However, these animals showed a change in their vocal repertoire and an altered acoustic structure in their communicative “social” calls, as well as permanent anatomical changes in the amygdala (AMY) and periaqueductal gray (PAG).

Results

We studied the vocal behavior in 10 infant marmosets (five males and five females) from five different sets of unrelated parents. In five of the neonatal animals, we performed surgical excitotoxic lesions bilaterally to the rostral portion of the dorsal ACC (24a and 24b) (**Fig. 1A, B**, [see Methods](#)). Starting seven days before the surgery and continuing until six postnatal weeks, infant vocalization behavior was recorded in an isolated, temperature-controlled incubator in 5 min sessions, 2-3 times a week (**Fig. 1C, 1D**). In four of the animals, the estimate of ACC volume from T2-weighted MR scans performed under anesthesia approximately eight months of age revealed a 60% decrease in ACC volume compared with four control animals (**Fig. 1E**; two animals were not scanned). Following sexual maturity, at approximately two years of age, the animals were euthanized, and their brains were histologically visualized to verify the extent of the lesion. We also examined downstream effects of the lesion, including an evaluation of its effects on mature neurons (NeuN), inhibitory neurotransmitters (GABA and GAD67), glial cells (GFAP and Iba1) and fiber tracts (myelin; **Fig. 1F**). See Materials and Methods for details.

Verification of the ACC lesion and its impact on downstream vocal structures

The intended lesions and reconstructed ACC damage based on histological evaluation are shown in one hemisphere for four animals in **Figs 2A** and **2B**, respectively. The ACC lesion covered most of the target cytoarchitectonic areas 24a and 24b of Paxinos et al.³⁰, just above the corpus callosum. The rostral limit of the lesions was adjacent to the genu of the corpus callosum and the caudal limit just anterior of area 23a caudally. Dorsally, the lesions extended past 24b into motor area 6M. There was little if any encroachment into subgenual area 25. Apart from one case which showed some sparing of the lesion in the left hemisphere, there was extensive overlap in the placement of the ACC lesion. The anterior-posterior extent of the bilateral lesion for each monkey is compiled in supplemental **Fig. S1** as sections from MRI images.

Representative photomicrographs of the ACC lesion and a control are presented in **Fig. 2B**, which shows the distribution of myelinated fibers in the ACC region stained using a high-resolution Black-Gold II myelin stain (Histo-Chem Inc., Jefferson, AR). There was clear evidence that the lesion created a major disruption to the normal radial arrangement of the fibers in the ACC region caused by extensive demyelination of the axons (**Fig 2B**). The ACC lesion also impacted the integrity of white matter tracts local to the site of the lesion (**Fig. S2**), but the transverse diameter of major fiber tracts, namely the corpus callosum and the anterior commissure did not differ between the groups. The loss of neurons, however, and the respective increase in glial cells at the lesioned site especially at the interface between the gray and white matter was clearly observed (**Fig. 2C, D**).

We examined the cellular and neurotransmitter composition of the amygdala (AMY) and periaqueductal gray (PAG), as these structures are downstream from the ACC and their natural development may be affected by the infant ACC lesions. We first investigated whether neurons in these structures were degenerated using Fluro-Jade C (Histo-Chem Inc.), which is used as a marker for apoptotic, necrotic, and autophagic cells. There was no sign of neurodegeneration in these downstream brain regions two years following the infant lesion. We next examined the proportion of neurons in the AMY and PAG expressing GABA, since changes in the relative number of inhibitory interneurons could serve as a marker for downstream neuroplasticity in response to the ACC lesion.³¹ We found a significant reduction of GABA positive neurons in two structures,

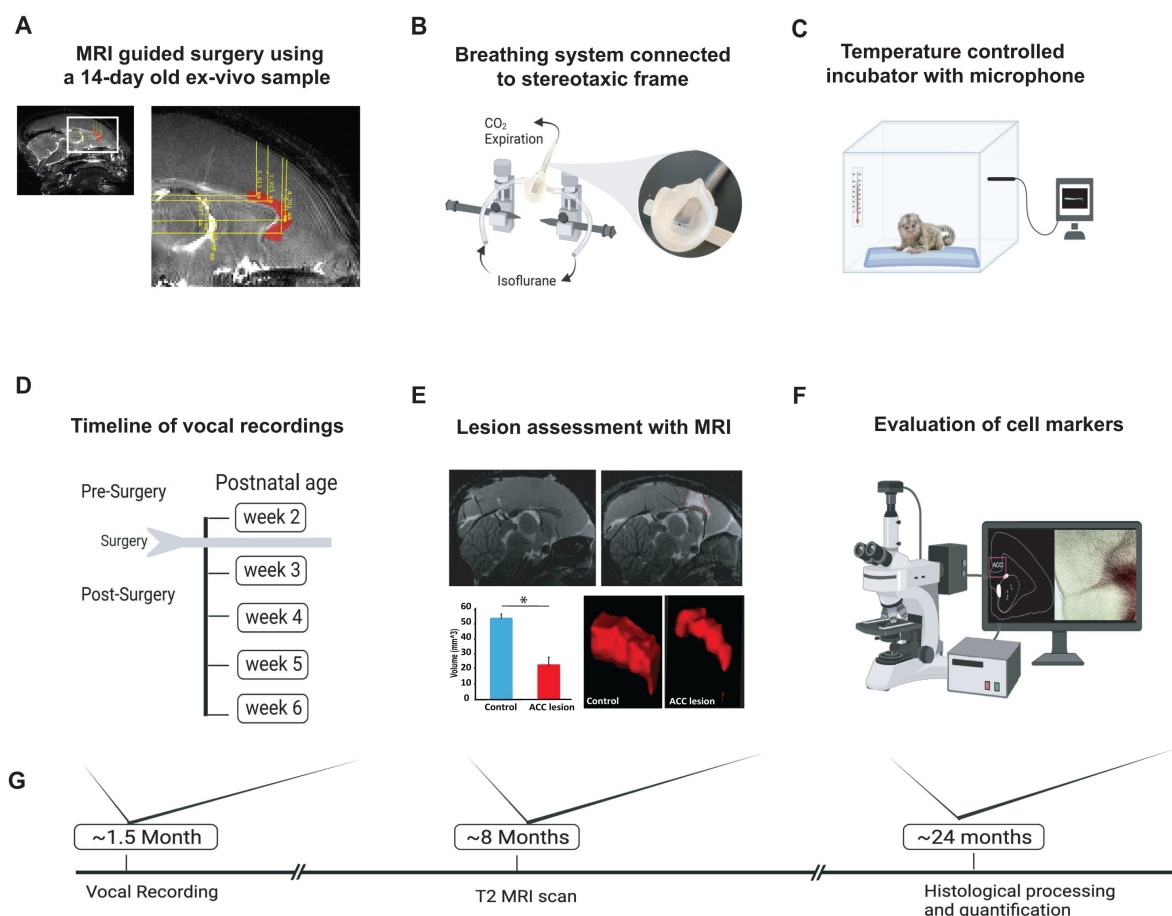


Fig. 1.

Experimental Design and timeline.

(A) Reference MR scan was obtained using an ex-vivo sample of a 14-day old marmoset. Parasagittal view of the reference scan shows location of injection coordinates targeting the rostral portion of the dorsal ACC (24a and 24b), bilaterally (red). **(B)** Gas anesthesia was supplied through a custom-made breathing system comprising a facemask fitted with a palate bar with a 0.6mm diameter hole. The palate bar was connected to a vital monitor to accurately detect small tidal end volumes during anesthesia while the animal was secured in the stereotaxic frame. **(C)** Five-minute vocalization recordings were obtained from infant placed in a softly padded temperature-controlled incubator. **(D)** Timeline of vocal recordings from postnatal week 2 to postnatal week 6. The ACC lesion was conducted at postnatal week 2 when animals were 14-16 days old. **(E)** Representative sagittal view of postoperative T2-weighted MR images of a control (left panel) and lesioned (right panel) infant to reveal extent of white hypersignal, which reflects edema due to injections of the excitotoxin and therefore approximate site of the ACC lesion. There was a significant reduction in total ACC volume in the ACC group relative to controls ($n=4$ /per group; $F(1,6) = 82.78$, $p<0.0001$). A representative 3-dimensional view of area 24 is presented showing the reduced volume of the ACC (right panel) relative to the normal volume in the control (left panel). **(F)** Schematic illustration highlights the end point of the experiment involving histological processing and evaluation of cell markers. **(G)** Longitudinal timeline shows approximate age of animals following vocal recordings, MRI lesion assessments and histological processing.

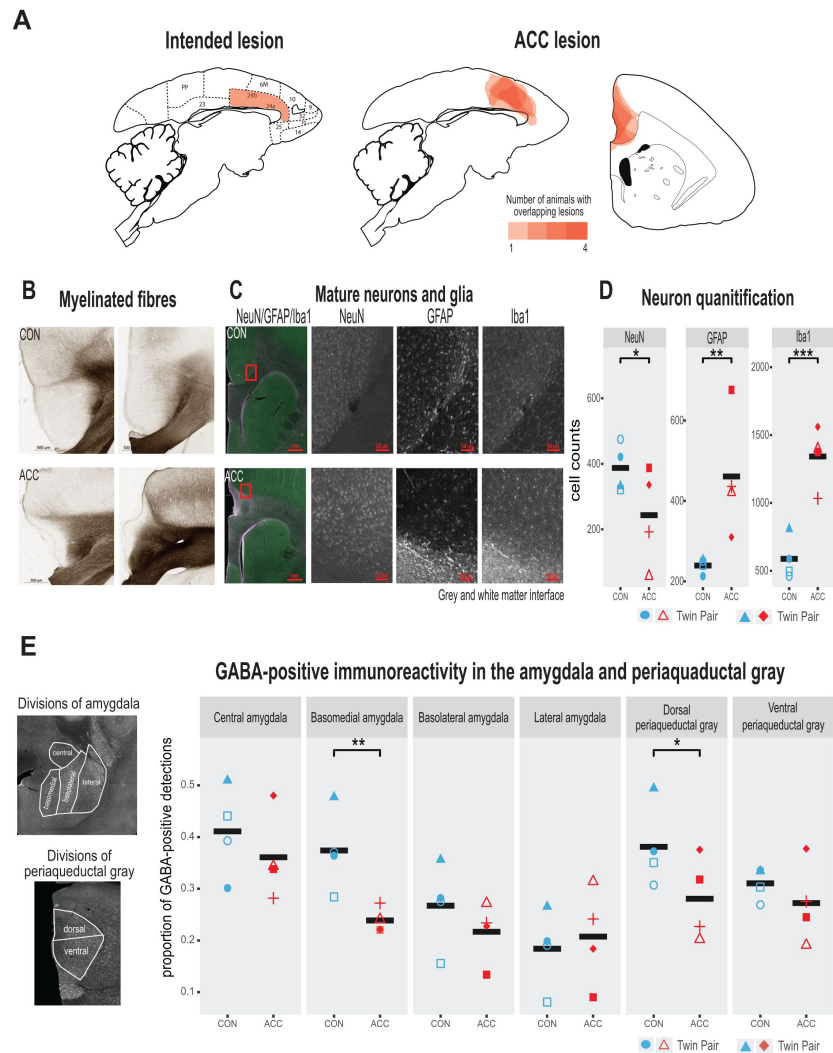


Fig. 2.

Lesion verification and impact of early life ACC lesion on vocal downstream structures

(A) Left panel shows a sagittal section from the standard marmoset brain depicting the intended ACC lesion shaded in red. The right panel shows schematic lesion reconstructions superimposed on a sagittal and coronal marmoset brain section depicting the extent of the ACC lesion shaded in red. Regions that appear darker indicate greater overlap in the damage present among different animals. **(B)** Magnified images of area 24 stained to visualize myelinated fibers in a representative control (top panel) and ACC-lesioned (bottom panel) animal. The normal radial arrangement of the myelinated fibers is disrupted following the ACC lesion. **(C)** Histological visualization of mature neurons and glia in representative control (top row) and ACC-lesioned animal (bottom row). Leftmost image shows neurons (green), astrocytes (violet) and microglia/macrophages (white) in the same image. Red square represents the magnified grayscale sections showing cell loss (NeuN), high levels of astrocytes (GFAP) and microglia/macrophages (Iba1) surrounding the lesion site at the grey and white matter interface in the ACC-lesioned animal (bottom row) relative to the controls (top row). **(D)** Histological quantification of mature neurons and glia in cortical area 24 in the ACC-lesioned animals, or in the corresponding intact cortical tissue bordering white matter in the controls. There was a reduction in the number of mature neurons (NeuN) and an increase in glia (GFAP and Iba1) in the ACC group relative to controls. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.00001$. **(E)** Left panel shows grayscale images with anti-NeuN staining depicting divisions of AMY and PAG where relative distribution of GABA-positive immunoreactive expression were quantified. Right graphs show the proportion of GABA expression in each division depicted in the AMY and PAG. Each circle represents one animal (ACC lesioned animal is red, Control animal is blue). Mean expression is represented by black bar. Data for two animals in ACC group overlap for basomedial AMY quantification. GABA-positive immunoreactivity was significantly down in the basomedial AMY and dorsal PAG.

the basomedial AMY and the dorsal portion of the PAG (**Fig 2E**). This reduction suggests a disruption of the normal inhibitory balance within the vocal network following the infant ACC lesions.

Vocal behavior persists immediately following neonatal ACC lesions

In the weeks following bilateral ACC lesions, infants tested in the isolated chamber continued to vocalize readily, which is somewhat surprising given the critical role of this structure in normal vocal behavior^{12,14}. From (presurgical) postnatal week 2 to (postsurgical) postnatal week 6, we annotated 23,000 calls from the five lesioned and five control marmosets. Sample spectrograms from audio recordings of a twin pair before and after surgery are shown in **Fig. 3A-B**. While there was variability among individuals, the calls were complex and diversified from postnatal week 2, consisting of cries as well as immature versions of adult vocalizations including phee, twitter, and trills, as well as complex calls when two calls merged such as trill-twitter or cry-phee, or any other combination (**Fig 3A**). By the sixth postnatal week, the call repertoire for both the lesioned animals and the controls had both evolved, with no conspicuous difference between groups (**Fig 3B**). Most notably, the relative reduction of the total rate and diversity of calls was similar between groups (**Fig. 3C**, $\chi^2(2)=2.8464$, $p=0.24$), with the reduction matching the known maturational changes accompanying growth of the vocal apparatus and increased respiratory powers³². Further analysis, on the proportion of calls emitted following surgery showed that the ACC lesion had minimal effects on the rate of most call types during this period (phee ($\beta = -0.07$, 95% CI [-0.31, 0.17], $p = 0.49$); twitter ($\beta = -0.07$, 95% CI [-0.16, 0.01], $p = 0.09$); trill ($\beta = -0.03$, 95% CI [-0.11, 0.06], $p = 0.49$); cry ($\beta = 0.13$, 95% CI [-0.03, 0.29], $p = 0.10$) (**Fig 3D**). The exception to this rule was an elevation in the rate of ‘other’ calls, which comprised tsik, egg, ock, chatter and seep calls. These calls were significantly elevated in animals after the ACC lesion ($\beta = 0.11$, 95% CI [0.03, 0.20], $p = 0.018$). This was driven mostly by an increase in the lesion group during postnatal week 4.

Two additional variables relatively unaffected by the ACC lesion were the call durations and inter-call intervals, acoustic features that have been used to track vocal development in previous studies^{4,6,33}. Consistent with the overall decrease in vocalization rate with increasing age, there was an associated increase in inter-call intervals which was noted at late postnatal weeks ($\beta = 0.33$, 95% CI [0.22, 0.43], $p < .001$) which held true for both controls and lesioned group ($\chi^2(2)=1.88$, $p=0.39$). None of the specific call types exhibited developmental changes in call type duration (phee ($\chi^2(2)=1.08$, $p=0.58$); trill ($\chi^2(2)=2.87$, $p=0.24$); twitter ($\chi^2(2)=2.79$, $p=0.25$); cry ($\chi^2(2)=0.057$, $p=0.97$), but there were slight changes in the duration of phee syllables exhibited only by animals with an ACC lesion which we discuss later. Importantly, we did not observe ACC-lesion induced changes in physical growth factors such as body weight and grip strength which could feasibly impact vocal parameters such as duration⁴ (**Fig. S3**).

Neonatal ACC lesions prevent early maturation of social contact calls

Whereas many of the basic vocal parameters evolved normally in the animals with the ACC lesions, one major difference related to their use of social contact calls. By 6 weeks of age, marmoset vocalizations are known to approach their mature state and become dominated by social contact calls, namely phees, trills, and twitters. The phee call is studied most extensively as a long- distance contact call. It is typically evoked when the animal is socially distanced or isolated, and it promotes vocal exchanges between marmosets located out of sight in far-away locations^{34,35} to facilitate reunion with family groups³⁶. Trills and twitters are short distance contact calls thought to monitor the presence of group members^{37,38}. Since the ACC

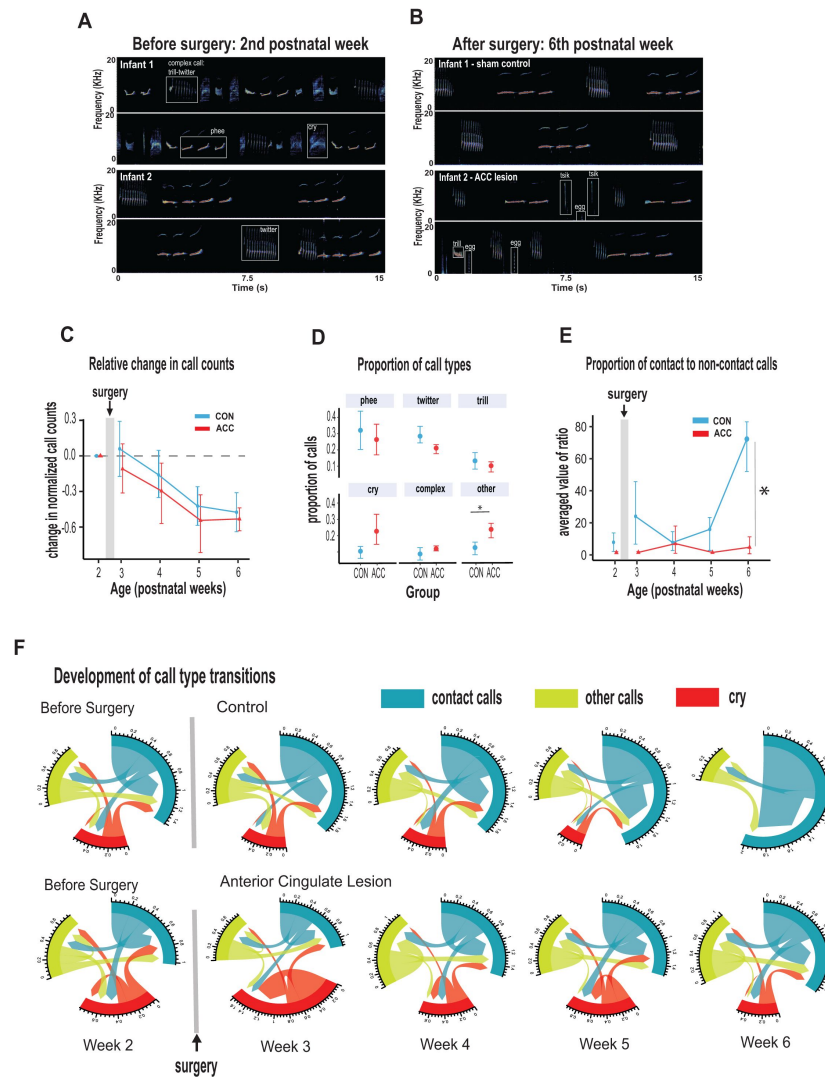


Fig 3.

ACC in early life is integral to postnatal development of social contact calls

(A-B) Spectrograms show sample 30-second vocal recordings of a representative control and ACC-lesioned marmosets before (postnatal week 2) and after surgery (postnatal week 6). Before surgery, the infants ‘babbed’ by emitting a wide range of immature concatenated calls, each with its own spectrogram motif illustrated and labeled in boxes. After surgery, at postnatal week 6, calls show reduced variability separated by distinct gaps or inter-call intervals. **(C)** Both groups show a reduction in the relative call count with increasing age. **(D)** Average proportion of each call type pooled from week 3 to week 6 following surgery. Animals in both groups were able to emit calls of different call types. Those with ACC lesions made minor calls designated as ‘other’ more frequently than controls but all major call types were produced at equivalent rates. **(E)** Proportion of social contact calls relative to non-social contact calls. The y-axis represents the averaged value of the ratios of the number of social calls divided by the number of non-social calls (x (# social calls / # non-social calls)). Despite their ability to produce all call types, the proportion of social contact calls comprising phoe, twitter and trills, was substantially reduced in animals with early life ACC lesions at postnatal week 6. Due to factors beyond our control (Covid-19), the number of recordings varied between animals: week 3: CON n=5, ACC n=5; week 4: CON n=5, ACC n=4; week 5: CON n=4, ACC n=3; week 6: CON n=4, ACC n=3. **(F)** Chord diagrams illustrate the likelihood of transitioning between call types. At 6 weeks of age, animals with ACC lesions showed a higher likelihood of transitioning between all call types, but less frequent transitions between social contact calls relative to the sham group. The chord diagrams visualize the weighted probabilities and directionality of these transitions between different call types. Weighted probabilities were used to account for variations in call counts. The thickness of the arrows or links indicates the probability of a call transition and the numbers surrounding each chord diagram represent the relative probability value for each specific transition.

plays a major role in socioemotional cognition (for review, see Devinsky et al.³⁹), we surmised that the ACC lesion might specifically influence the socioaffective content of the vocalization that is normally expressed through contact calls.

We thus grouped phee, twitter, and trill calls as social contact calls and compared them with non-contact calls (i.e., cries and other minor occurring calls like tsik, egg, ocks, chatter, and seep). At 6 weeks of age, social contact calls predominated the control animal's vocalization. However, in ACC-lesioned animals, this aspect of social vocal behavior was substantially reduced. This difference emerged gradually after the surgery, and was only evident at 6 weeks of age (**Fig 3E**; $\chi^2(2)=7.58$, $p=0.022$). By the sixth week, the social vocal repertoire of the lesioned animals was altogether different from the control animals, with a much smaller proportion of social contact calls.

To further understand the effect of the ACC lesion on the normal distribution of calls, we investigated the call transition probabilities between contact calls, cries, and other calls (**Fig 3F**). In contrast to the control animals, whose repertoire was dominated by social contact calls, the ACC lesion group showed frequent transitions mostly to other non-contact call types (u-index Wilcoxon test, $p = 0.055$). These data suggest, therefore, that the ACC mediates developmental changes within the first 6 weeks of life that lead to the dominant production of isolation-induced contact calls and the gradual reduction of cries and other calls.

Neonatal ACC lesions alter sequential characteristics of social contact calls

We examined the characteristics of vocal sequences to learn more about how early life ACC lesions might influence the acoustic signals that marmosets potentially relay to distantly located family members or other conspecifics when socially isolated. We focused on phee syllables, which are discrete elements or components of a call separated by very short intervals^{39,40}. Thus, a sequence of phee calls may comprise multiple syllables (**Fig 4A**). The functional significance of syllables is not clearly understood but a change in the number of syllables or their acoustic characteristics might feasibly alter the message conveyed to a family that cannot be seen or heard. This is especially important to young infant monkeys that are naturally demanding of attention, and even more so if isolated. The number of syllables per phee call was highly variable amongst the animals, ranging from 1 to 8 syllables per phee.

The ACC lesion did not greatly affect the phee syllable count. Aside from a transient decline in the number of syllables in the week after the surgery (postnatal week 3: Wilcoxon test $p=0.042$), these animals showed the normal preferred range of 3-4 phee syllables at later postnatal weeks (**Fig 4B**). Even in the sixth postnatal week, when the proportion of phee and other contact calls was much lower in the ACC lesioned animals, the number of syllables in those phee calls that were issued was similar to the control group.

However, other phee call variables were affected by the lesion. For example, the duration of phee syllables was shortened in ACC-lesioned animals ($\chi^2(5)=13.27$, $p=0.021$), particularly in the later syllables of a multi-syllabic phee. This effect emerged gradually and was most pronounced when the animals were 5-6 postnatal weeks (**Fig. 4C**). Likewise, the amplitude of phee syllables was also affected by the ACC lesion ($\chi^2(5) = 48.178$, $p<0.0001$) with lesioned animals making louder phee calls (**Fig 4D**). For each phee syllable, the amplitude difference between groups increased until postnatal week 5 and then disappeared at postnatal week 6 (postnatal week 4: $\beta = 3017.75$, 95% CI [1831.61, 4203.89] $p < .001$; postnatal week 5: $\beta = 3719.00$, 95% CI [2389.88, 5048.12], $p < .001$). In line with the increase in amplitude, the peak frequency of each phee syllable was also lowered by the ACC lesion (**Fig 4E**). This change occurred soon after the lesion [postnatal week 3: $\beta = -488.63$, 95% CI [-702.40, -274.85] $p < .001$; postnatal week 4: $\beta = -384.61$, 95% CI [-607.98, -161.24], $t(513) = -3.38$, $p < .001$].

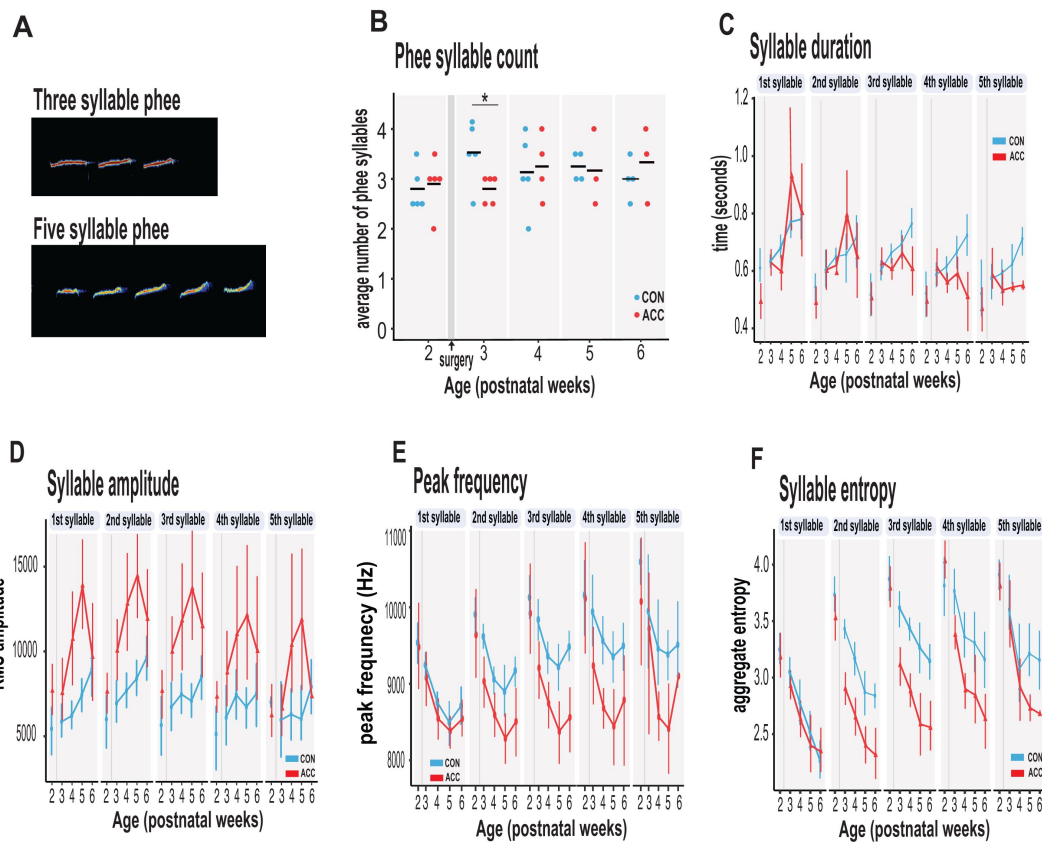


Fig. 4.

ACC lesion alters structural characteristics of long distance social phee calls

(A) Sample spectrogram with examples of three and five syllable phee calls **(B)** The ACC lesion caused a reduction in average phee syllable counts immediately after the ACC lesion (red dots) at postnatal week 3, but then normalized to 3-4 syllables thereafter. **(C)** Phee syllable duration ACC-lesioned group became shorter for multisyllabic phee ≥ 3 especially with increasing age. **(D)** The effective amplitude for each phee syllable increased for the ACC group until postnatal week 6. **(E)** Animals with ACC lesions emitted low entropy phee for calls as low as 2-syllables and continued until postnatal week 6. **(F)** Decrease in peak frequency of phee calls immediately following ACC lesion. Data in D-F represents average of specific syllable in a phee sequence irrespective of the number of syllables in a phee (e.g., 1 syllable phee, the 1st syllable in a 2-syllable phee, in a 3-syllable phee and in a 4-syllable phee). Due to factors beyond our control (Covid-19), the number of recordings varied between animals: week 3: CON $n=5$, ACC $n=5$; week 4: CON $n=5$, ACC $n=4$; week 5: CON $n=4$, ACC $n=3$; week 6: CON $n=4$, ACC $n=3$. Error bars are confidence intervals. Gray shaded lines or bars represent time of surgery.

Finally, we examined entropy of phee syllables as a measure of vocal complexity⁷². High entropy in multisyllabic phees would indicate that these vocalizations are diverse, variable and unpredictable. We found that animals with ACC lesions exhibited lower entropy in phee syllables relative to controls as early as postnatal week 3 ($\chi^2(5) = 34.528$, $p < .0001$) (**Fig 4E**) suggesting that their vocal sequences were simple, less diverse and stereotyped. Together, our data suggest that the ACC lesion compromised the normal development of the phee signature for each monkey by making them shorter, louder, and monotonic.

Discussion

We found that early life ACC lesions led to rather specific alterations in the production of vocal calls, developmental changes to the quality of social contact calls, and their associated sequential and acoustic characteristics were compromised. Contact calls that normally dominate the marmoset vocal repertoire at around 6 weeks were selectively diminished in the lesioned animals. When one common contact call, the phee call, was issued, its structural characteristics were unusual. The ACC lesions also led to permanent changes in remote brain areas known to be involved in vocal behavior. Notably, the proportion of presumptive inhibitory interneurons was reduced in both the basomedial AMY and dorsal PAG. We can infer from these findings that an intact ACC in early life is integral to postnatal development of social vocalizations, and that its interactions with vocalization-eliciting sites from a very early age is fundamental to the normal vocal expression of social behavior.

Consistent with previous reports, the range of call types observed in both neonatal controls and neonatal ACC-lesioned animals within the first weeks of postnatal development were stereotyped and repetitive^{33,41}. With increasing age, the call rate gradually declined such that by the time the animals were six weeks of age, the most common vocalizations were those that conveyed social distance. Such calls solicit attention from family members and may trigger a range of behaviors including search, approach, interaction, and caregiving in response to the need for social contact. This is especially so for infants whose well-being depends on social feedback and reciprocal interaction^{4,5,35,42}. Our data suggest that the ability to effectively convey this social need was significantly altered in animals with early life ACC damage. At 6 weeks of age, these animals were not making social vocalizations at the same high rate as their age-matched controls. This reduction in social vocalizations does not appear to reflect a general slowing in vocal maturation, since other call types had advanced at the normal rate.

Since ACC lesions in humans cause social apathy^{43,44}, one possibility is that early life ACC removal altered the animals desire or motivation for social reinforcement; these infants appeared to make little effort in using vocalizations to solicit social contact when socially isolated. This change in call usage aligns with their social development period at around postnatal week 6 when infant marmosets transition from using fixed, stereotypical calls to a flexible and more individualized call repertoire as they wean towards independence³³. Our data suggest that this transition does not occur normally following an ACC lesion.

In addition, the neonatal ACC lesion altered the quality of the infants' long-distance phee calls; they were shorter in duration, louder in amplitude, lower in peak frequency and abnormal in their entropy such that the acoustics of calls were blunted of variation and less diverse. This suggests that the social message conveyed by these infants to their families through phee calls, even though it was loud and propagating over long distances, was potentially deficient, limited, and/or indiscriminate. However, the impact of entropy on emotional quality of vocalizations has not been systematically explored. Generally speaking, high entropy relates to high randomness and distortion in a signal. Accordingly, one view posits low-entropy phee calls represent mature

sounding calls relative to noisy and immature high-entropy calls⁴². In the current study, the reduction in syllable entropy observed for both groups of animals with increasing age is consistent with this view.

At the same time entropy relates to vocal complexity; high entropy refers to complex and variable sound patterns whereas low entropy sounds are predictable, less diverse and simple vocal sequences⁷². One possibility is that call maturity does not equate directly to emotional quality. In other words, a low-entropy mature call can also be lacking in emotion as observed in humans with ACC damage; these patients show mature speech, but they lack the variations in rhythms, patterns and intonation (i.e., prosody) that would normally convey emotional salience and meaning. Our observation of a reduction in phee syllable entropy in the ACC group in the context of being short, loud with reduced peak frequency is consistent with this view and suggests that the ACC group were emitting phee calls that were potentially lacking emotional meaning.

The long-term behavioral implications of such imperfect vocalizations is currently unknown but could, ostensibly, affect their ability to use long distance social vocalizations to maintain intragroup functions such as warn of predators, strengthen family bonds, and maintain group cohesion more generally². Since the ACC exerts regulation over autonomic responses^{45–47}, its ablation so early in life might feasibly blunt respiratory and vocalization responses in negative emotional environments such as social isolation. How these factors impact vocal behavior is a current topic of investigation (Sheikhbaheei et al., *SfN abstracts*. 2023).

Although we found that an intact ACC in early life is integral to the postnatal maturation of social vocalizations, we also show that it is not critical for production of infant vocalizations more generally. A number of early observations reporting the loss of learned or spontaneous vocalizations following bilateral ACC lesions, left this question open, though it has been clear that vocal production in adults can withstand ACC damage^{16,17,48–50}. In addition to ablating the ACC, researchers found that it was necessary to ablate other frontolimbic areas to permanently eliminate infant vocalizations such as cries^{17,29}. Our findings indicate that innate vocal production in the earliest phases of life, as early as two weeks postnatally in the marmoset, is not critically dependent on the ACC. The infant ‘babbling’ behavior observed in marmosets and other primates^{4,41,51} was largely preserved in the ACC-lesioned infant monkeys which, like the control group, produced long sequences of concatenated calls composed of rudimentary features of mature adult-like calls.

While the ACC is not essential for infant vocal behavior, its absence affects not only the maturation of social vocal behavior, but also the anatomical compositions of structures with which it is interconnected. We noted a decrease in presumptive inhibitory interneurons in the dorsal PAG and basomedial AMY, two prominent ACC target regions involved in vocal behavior^{7,20,52}. We can speculate that this reduction might stem from a prolonged deafferentation of cingulate inputs, gradually leading to a rebalancing of the excitatory/inhibitory elements in the local circuit.

One potentially related observation is that phee calls became louder in the weeks following the surgery. It is interesting to speculate that such amplitude increases might reflect a local decrease in inhibition in structures such as the AMY or PAG, whose activity is thought to tune the emotional characteristics of social vocalizations. The primate ACC receives dense projections from the basomedial AMY with notably minimal direct input from the lateral nucleus^{53–55}, and layer V pyramidal neurons project to the PAG^{56,57} with a greater concentration directed to the dorsolateral column⁵⁸. Thus, the ACC has the capacity to directly activate the brainstem vocalization pathway as early as the first few weeks of life. Both, the AMY and PAG are highly active during contexts in which threat related vocalizations would normally be triggered^{59–61}, and both regions elicit vocalizations through electrical stimulation or pharmacological disinhibition^{12,62–64}. We cannot be sure when during postnatal development the ACC lesion

altered GABA expression in the AMY and PAG, but from our results, we can infer that the appropriate modulation and coordination of social vocal behavior requires the normal postnatal development of the ACC.

Existing evidence in monkeys and humans demonstrate unequivocally the importance of the ACC in its contribution to emotional vocalization. In humans, as in monkeys, ACC lesions do not eliminate vocal behavior, but instead tend to remove the intonation and prosodic features of the vocalization characterized as expressionless⁴⁹. This is consistent with the changes observed in the marmoset phee calls. In general, our data suggest that the ACC shapes the emotional structuring of social calls during the first few weeks of life in the marmoset. Given the many similarities to humans, and the strong contribution of socioemotional information to the vocal productions beginning in infancy, it is reasonable to speculate that similar mechanisms apply to the development of early life human vocal behavior. Importantly, our data confirm the importance of vocalizations as a means of conveying social information even when family members or conspecifics are not physically present. Our data suggest that in the absence of a functioning ACC in early life, infant calls conveying social information that would elicit feedback from parents and other family members may be compromised, and this could potentially influence how that infant develops its social interactive skills. The ability to normalize brain circuits in early life would provide a major therapeutic advance for the remedial treatment of social deficits that plague disorders of mental health.

Materials and Methods

Subjects

All procedures accorded with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the National Institute of Mental Health. A total of 10 marmosets (*Callithrix jacchus*), 5 males and 5 females, all born in captivity, were used in this study. Five infants received ACC lesions at 14-16 postnatal days old. Five others served as age-matched controls. The infants were raised by parents and siblings in family groups comprising 4-6 members and housed in temperature-controlled rooms (~27°C), 50-60% relative humidity under diurnal conditions (12h light:12h dark). Food and water were available ad libitum, supplemented with fresh fruit or vegetables. One animal showed sparing of the ACC lesion in one hemisphere. The final sample size for the behavioral data was n=5/per group. For the MRI and histological data, the final sample size was n=4/per group.

Surgery

We first obtained a reference MRI scan using a 14-day old ex-vivo sample (Fig. 1A). A T2 weighted scan was obtained using 7T Bruker Biospin MRI platform with an eight-channel volume coil. Using ParaVision Acquisition 6.0.1, the following echo sequence was used to acquire a 3-dimensional volume of the infant marmoset brain: TR = 400, TE = 72ms, flip angle= 90 degrees, matrix size = 256x256x214, resolution = 0.15 mm isotropic, number of averages = 8, number of repetitions =1) and the total scan time was 3 hrs. The scan was aligned horizontally by rotating the image until the anterior and posterior commissures were positioned at the same height and water filled ear bars were used to obtain the interaural reference. We then used ITK-SNAP⁶⁵ (Yushkevich et al., 2006) to identify the anterior cingulate cortex (ACC) at the coronal planes before the genu of corpus callosum to the level of anterior commissure. The coordinates were calculated relative to the ear bars and midline references, both of which were visible on the scan. The resulting 14-day old marmoset scan served as a template atlas to calculate injection coordinates to target the rostral portion of the dorsal ACC (24a and 24b), bilaterally, in all marmosets. We calculated 5 injection coordinates for each hemisphere: (1) AP: 10.7 mm, ML: ±0.7 mm, DV: -2.9 mm; (2) AP: 10.7 mm, ML: ±0.7 mm, DV -4.5 mm; (3) AP: 9.5 mm, ML ±0.7 mm, DV -3.5 mm; (4) AP 8.5 mm, ML ±0.7 mm, DV -3.6 mm; (5) AP 7.5 mm, ML ±0.7 mm, DV -3.5 mm.

The entire surgical procedure was performed under aseptic conditions in infant monkeys that were 14-16 days old. During surgery, monkeys received isotonic fluids. Heart and respiration rates, body temperature, blood pressure, and expired CO₂ were monitored throughout the procedure. Pre- and post-operatively, monkeys received non-steroidal anti-inflammatory drugs, (meloxicam, 2mg/ml, s.c) to reduce swelling. The monkey was first immobilized with an anesthetic dose of alfaxalone (10mg/kg, i.m) combined with diazepam (5mg/kg, i.m). In this state, the infant's head was shaved, and vital electrodes were secured on the infant's chest. Temperature was measured with a rectal probe. The infant's head was then secured in a small animal stereotaxic frame (Stoelting Company, Illinois) attached to a custom-built stage fitted with eye bars, ear bars and a pallet bar to accommodate the small head. Once the head was secured in the frame, anesthesia with isoflurane gas (1-2% to effect) was provided through the custom fitted mask (**Fig. 1B**). An integral part of the pallet bar was a gas hole (0.6 mm diameter) that ran along the length of the bar and connected, via tubing, to a vital monitor to measure small end tidal CO₂ volumes.

Following a midsagittal incision, the scalp was retracted, and a craniotomy was made above the target coordinates of the brain. A 5 µl syringe (33 gauge, Neurosyringe, Hamilton Company, Reno, NV) was used to administer bilateral injections of 0.12M NMDA (M3262, Sigma-Aldrich) dissolved in sterile filtered saline into the anterior cingulate cortex (0.5 µl per injection site). Each injection was made over 2 mins and the injector remained in place for an additional 4 mins for dispersion. When all injections were complete, the scalp was closed with intradermal absorbable sutures and the infant was allowed to recover in an intensive care unit that was void of extraneous sensory stimulation (e.g., excessive bright lights and loud noise). During recovery, marmosets received a combination of Esbalic and Enfamil (3:1 ratio) infant formula every 2-3 hours. When fully awake, each infant was returned to its family unit. A total of five marmosets received the neonatal cingulate lesion. Another five marmosets served as controls: two received saline injections (shams), one received a craniotomy only, and another two were unoperated.

Vocal recordings

Each infant was placed in a temperature-controlled incubator set to ~38°C (Thermocare, CA), and the emitted vocalization was recorded for 5 mins. Sound recordings were acquired using a cardioid microphone (Sennheiser ME 64, Sennheiser, Wedemark, Hanover, Germany) that was placed on the side of the incubator (**Fig. 1C**). The microphone was connected to a computer and recorded sounds were digitized at a sampling frequency of 44 kHz using Raven Lite software (Cornell Lab of Ornithology, Ithaca, NY). Due to a variety of extraneous factors beyond our control including restrictions due to the COVID-19 pandemic, the exact day and number of recordings differed between monkeys. Therefore, recording sessions from each infant were grouped by week. All recording sessions were conducted without the presence of investigators in the recording room. The infant was then returned to its family unit.

Acoustic Analysis

The spectrogram of each audio file was obtained and visually inspected using Raven Pro 1.6 (Cornell Lab of Ornithology, Ithaca, NY). Spectrogram were generated with a Hann window of 512-sample points to filter the signal at 3dB bandwidth of 124 Hz (example, **Fig. 2 A-B**). The calls were manually classified by a defined classification system^{37,51}. To identify call types in the spectrogram of a recording, Raven software features, such as amplitude waveform, spectrogram and audio playbacks were used. Six major call types (phee, trill, trill-phee, twitter, cry, and complex calls) were identified from spectrograms, along with other minor call types (tsiks, chatter, egg, ock, seep). In some cases, when trill-pees and pheels looked similar in spectrograms of a recording, acoustic parameters such as entropy were used to carefully classify calls. Complex calls, comprised vocalizations with elements from at least two different simple call types such as trill-twitter or twitter-phee, etc. From each recording, call types were manually annotated by 3 trained

investigators (inter-rater reliability >80%). The spectrograms were used to obtain acoustic measurements such as peak frequency, RMS-amplitude and aggregated entropy and exported for further analysis.

Acoustic characteristics such as inter-call interval, syllable duration, number of phee syllables were quantified using a custom-written R script. Acoustic analyses were performed only for phees, which served as the major call type because of their abundance during postnatal weeks. In some cases, vocalization quantity and amplitude was largely suppressed for several minutes after handling by the experimenters. Consequently, analysis of each recording began after 2 minutes had elapsed.

Lesion Assessment with MRI

Gross ACC volume was measured from anesthetized MR scans performed at approximately 8 months of age. Anesthesia was induced with 5% isoflurane. The animals were then placed in an MR compatible cradle where its head was secured using ear bars. Isoflurane was maintained at 1.5 - 2.5% and vitals were monitored with V9004 Series Capnograph Monitor (San Clemente, CA). We obtained T2 weighted scan (n=4 Control and n=4 for ACC) using the MR procedure described above with the following echo sequence: TR = 30, TE = 48, matrix size = 144x144x128, resolution = 0.25 mm isotropic, number of averages = 8, number of repetitions = 1. The rostro-caudal extent of the ACC was segmented and measured using ITK-SNAP 4.0. A representative sagittal view of the lesioned and non-lesioned ACC can be seen in [Fig. 1E](#). Voxels containing ACC were carefully labeled from the anterior to posterior slice of the MR scan for each subject.

Histological preparation and quantification

At approximately 24 months of age, the marmosets were euthanized and perfused with 0.1 M PBS followed by 4% paraformaldehyde. The brains were extracted and cryoprotected in 0.1 M phosphate buffered sucrose (in steps of 10%, 20%, and 30% w/v). The brains were partitioned along the midline and right hemispheres were used for further histological processing after sectioning in coronal orientation on a sliding microtome and cryostat into 40 µm sections.

Immunohistochemistry

The immunohistochemistry (IHC) was performed on two series of free-floating sections. Initially, from each of the 8 brains, six sections each were collected around three rostrocaudal planes at the following approximate locations (in reference to the interaural axis): +13.30 mm (target brain region: the anterior cingulate cortex); +9.20 mm (target brain region: the amygdala); and +2.05 mm (target brain region: the periaqueductal gray matter). Subsequently, these 18 sections were divided into two IHC series of 9 sections each, where three sections covered each target brain area, for separate processing. The first IHC series was processed to visualize major cell classes that are likely to be affected by a brain lesion (neurons: primary antibody against NeuN, astrocytes: primary antibody against the glial fibrillary acidic protein (GFAP), microglia/macrophages: primary antibody against the ionized calcium-binding adaptor molecule 1 (Iba1). The second IHC series was processed to visualize and assess the ratio of GABA-ergic neurons to all neurons: such changes in the relative number of inhibitory neurons could indicate local downstream neuroplasticity in reaction to the ACC lesion, as GABA-ergic neurons are primarily responsible for local inhibition. In the second IHC series we visualized the distribution of NeuN, as well as of neurotransmitter GABA and GAD67, a rate-limiting enzyme in GABA synthesis that produces more than 90% of GABA in the central nervous system.

The first IHC series was incubated in a cocktail of the following primary antibodies for 60 h at 4°C: NeuN (chicken, ABN91, Millipore Sigma), 1:500 dilution; GFAP (goat, SAB2500462, Millipore Sigma), 1:1,500 dilution; Iba1 (rabbit, 019-19741, Fujifilm Wako Chemicals), 1:1,500 dilution). Then after several washes the sections were incubated in a cocktail of the following secondary antibodies for

2 h at RT: 1:200 donkey anti-chicken Alexa Fluor 488 (703-545-155, Jackson ImmunoResearch), 1:500 donkey anti-goat Alexa Fluor 594 (A21207, Invitrogen), 1:400 donkey anti-rabbit Alexa Fluor 680 (711-625-152, Jackson ImmunoResearch). Additional antibodies used in the second IHC series were as follows. Primary: GABA (rabbit, A2052, Millipore Sigma), 1:600 dilution; GAD67 (mouse, MAB5406, Millipore Sigma), 1:500 dilution. Secondary: 1:500 donkey anti-rabbit Alexa Fluor 594 (A21207, Invitrogen), 1:400 donkey anti-mouse Alexa Fluor 680 (A10038, Invitrogen). Finally, the sections were mounted on a glass slide, air-dried, and cover-slipped with DEPEX mounting media (13515, Electron Microscopy Sciences, Hatfield, PA).

Myelin staining

Myelinated fibers were stained with aurohalophosphate-based Black-Gold II (Histo-Chem Inc., Jefferson, AR) as shown in Schmued et al.⁶⁶ according to the manufacturer's protocol and cover-slipped with Permount (SP15, Fisher Scientific).

Staining for degenerating neurons

Fluoro-Jade C (Histo-Chem Inc.) stain was used to visualize degenerating neurons according to the manufacturer's protocol in the target brain regions of ACC, amygdala, and PAG. There was no observable signal of neurodegeneration in the studied regions.

Imaging and cell counting

Each histological section was digitized at 0.65 μm resolution ($10\times$ magnification) using a Zeiss AxioScan microscope slide scanner. Images were then split into a separate channel for each fluorophore. Cell detection and counting were done with an open source QuPath software 0.3.2.⁶⁷ As each fluorescence channel was analyzed separately, the loci of immunofluorescence that were counted do not necessarily correspond to unique cells, especially for microglia and GABA channels where the fluorescent signal was more diffuse in appearance. Cell segmentation in brain regions downstream to the lesion site was done using the random trees algorithm embedded into QuPath. Cell segmentation of the lesion site was done by custom-trained Cellpose models (doi: 10.1038/s41592-022-01663-4). Although the researcher performing cell counts was blinded to the marmosets' identity, it was possible to identify the site of the lesion and determine the animals group membership. Raw cell counts were transformed into cell densities per mm^2 to account for size differences in ROI areas.

Statistical analysis

All analyses were performed using R 4.2.3 (<https://www.R-project.org/>). For vocalization analysis, recording sessions were averaged by week and unless otherwise noted the data are represented as the mean values with mean $\pm$ confidence interval. Recording sessions obtained before the surgery were grouped as postnatal week 2 (pre-surgery) and all the recordings after the surgery were binned into postnatal weeks 3 to 6 (post-surgery). Due to COVID-19 related restrictions, recordings for some infants could not be extended beyond postnatal week 3.

Packages in tidyverse library⁶⁸ were used for data processing and analyses. Linear mixed effect models (LMM) were used to analyze postnatal datasets, and these models were fitted with the lmer() function in lme4 package⁶⁹. For estimation of coefficients, the maximum likelihood method was used. Models were fitted with postnatal weeks and experimental groups as fixed factors. To account for inter-individual variability, each monkey was modeled as a random effect. For multisyllabic phee analysis, syllables were nested within monkeys' random effect. Models with and without lesions were used to test the effect of ACC lesion and lesion effect is considered significant at an α of 0.05^{70,71}. Model assumptions were tested using the check_model() function available in the performance package. Log transformation was performed on some dataset to meet LMM model assumptions. When normality assumption was violated, non-parametric test (Wilcoxon test) was also used. Graphs were created using the package ggplot2.

For analysis involving immunofluorescence there were some inhomogeneities in the spatial distribution of IHC signal. The raw immunopositive detections for specific markers were normalized by NeuN cell counts that were obtained in the same Qupath processing pipeline. The final measure for each antigen-specific immunopositivity count was the ratio computed from the number of antigen-positive detections divided by the sum of the antigen-positive detections and NeuN-positive cells. This transformation bounded the possible antigen-specific detections between 0 and 1 and allowed for parametric modeling with beta distribution. Cell counts were fitted using glmmTMB software package in R statistical computing environment. As there were 3 sections per animal for each region of interest, these were modeled as random effects. Widths of major fiber tracts were modeled by a linear regression (function `lm` in base R). Final p values were adjusted for multiple comparisons with the false discovery rate method for each antigen.

Data Availability

All data needed to evaluate the conclusions in the paper are present in the paper.

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Additional information

Author Contributions (CRediT)

Conceptualization and methodology GN, YC; Software GN, DM, ACP, SPB; Formal analysis GN, DM; Investigation GN, DM, ACP; Resources YC; visualization GN, DM, YC; writing original draft GN, YC; Writing review and editing GN, DM, ACP, SPB, YC; Supervision DM, YC.

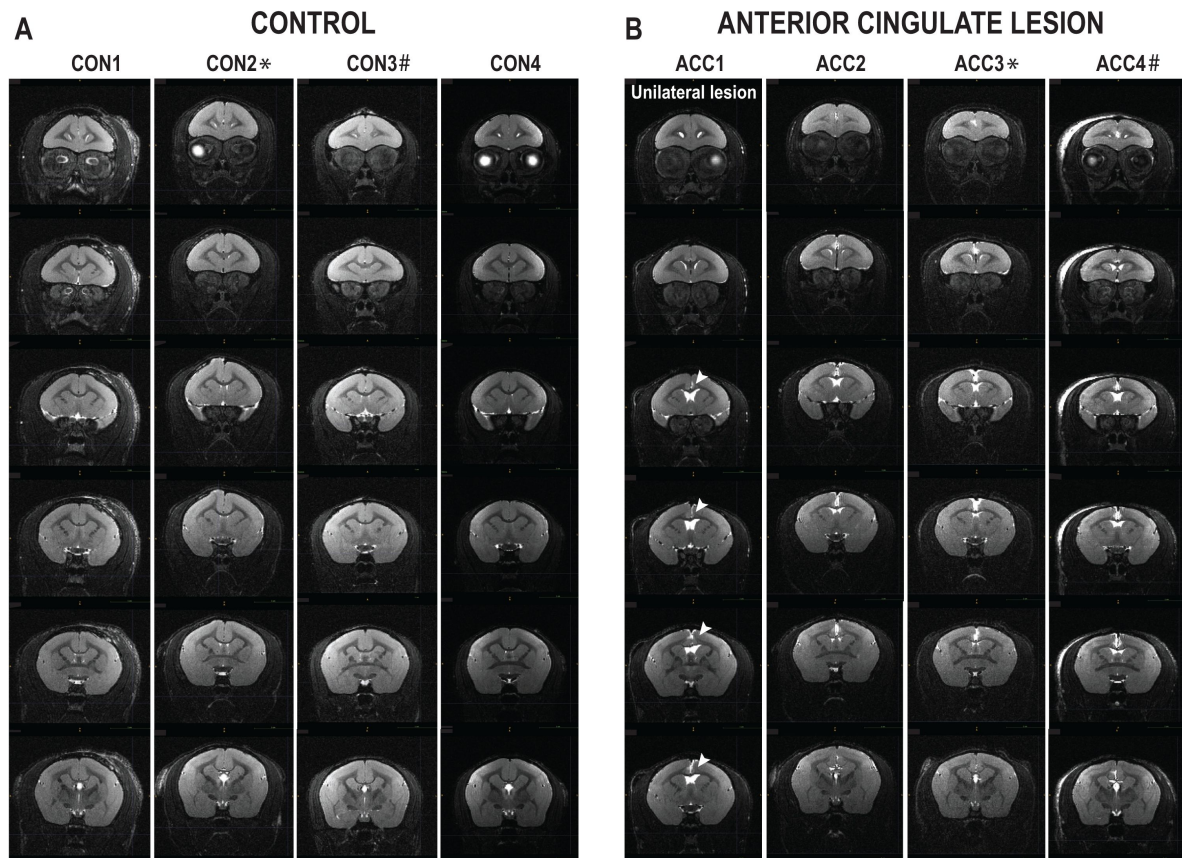


Fig. S1.

Bilateral MRI scans. Images from MRI scans of each marmoset representing coronal planes ranging from pregeniculate region until retrosplenial area. Each slice is 0.25mm apart. ACC1 shows a unilateral lesion depicted by the arrow. CON2* and ACC3* are twins, and CON3# and ACC4# are twins.

Fig. S2.

Representative images of white matter tract from a CON and ACC-lesioned subject. Both anterior and posterior corpus callosum are visibly narrowed in the area of the ACC lesion. The transverse widths of the white matter tracts were measured from sections stained for myelin at the following approximate rostrocaudal planes (in reference to the interaural axis): genu of the corpus callosum and rostrum of the corpus callosum (both +12.80 mm AP) were measured at the nadir of the overlying cortex; anterior corpus callosum was measured at the midline and the anterior commissure was measured at the medial juncture with the internal capsule (both +9.50 mm AP); posterior corpus callosum and fimbriae were both measured at the junction with each other (both +1.80 mm AP).

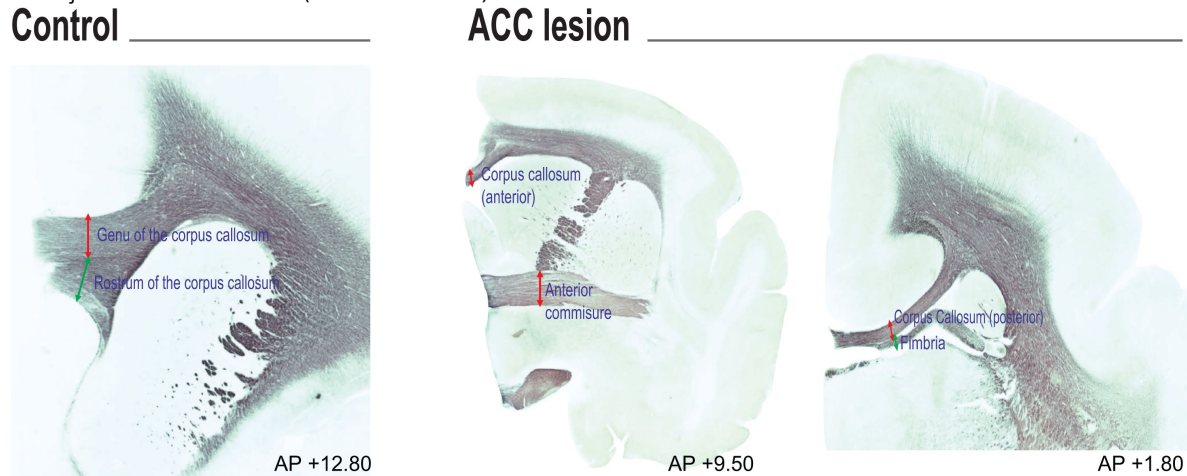
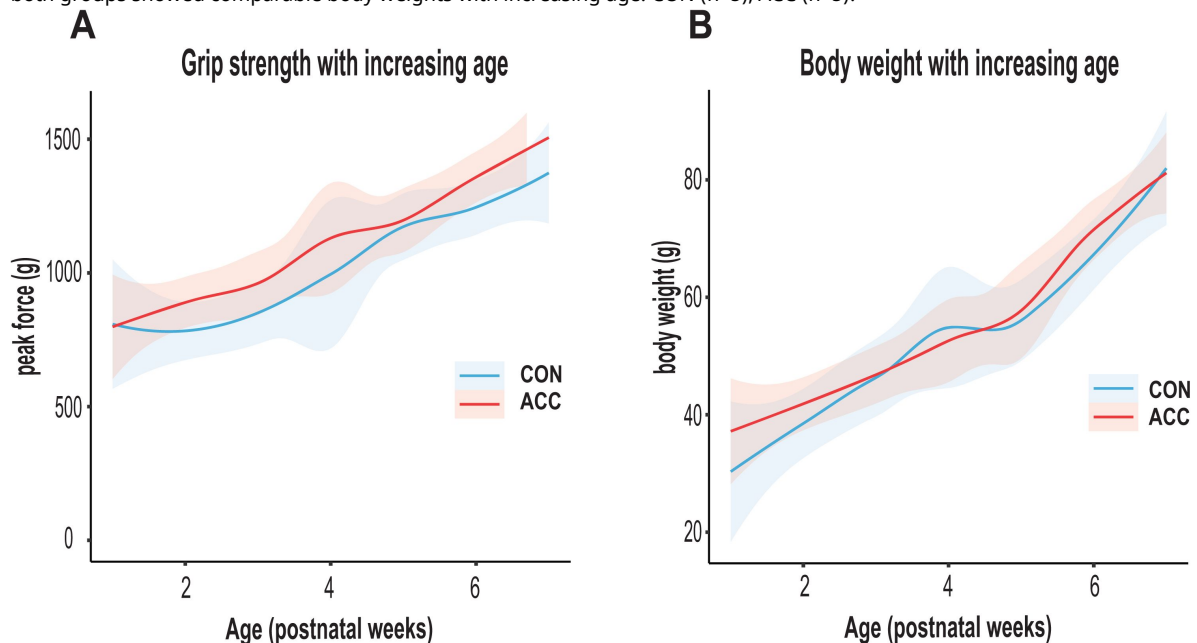


Fig S3.

Physical factors in developing marmosets.

(A) Muscular strength was quantified using the Bioseb (BIO-GS4) grip strength monitor. The infant was allowed to grip onto a bar while being gently pulled backwards in a horizontal plan to determine the maximal peak force (grams). The ACC lesion did not cause any changes to forelimb muscular strength. **(B)** Average body weight (grams) with increasing age. Animals in both groups showed comparable body weights with increasing age. CON (n=5), ACC (n=5).



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Reviewer #1 (Public review):**Summary:**

This study seeks to quantify changes in vocal behavior during development in marmosets with bilateral anterior cingulate cortex (ACC) lesions. The ACC and its role in social vocal behaviors is of great interest given previous literature on its involvement in initiation of vocalizations, processing emotional content, and its connectivity to two other critical nodes in the vocal network, the amygdala and the PAG. The authors seek to test the hypothesis that the ACC contributes to the development of mature vocal behaviors during the first few weeks of life by disrupting this process with neonatal ACC lesions. Imaging and histological analyses confirm the extent of the lesion and suggest downstream effects in connected regions. Analysis of call rates and call type proportions show no or slight differences between lesioned and controlled animals. Additional analyses on the proportion of grouped 'social' calls and certain acoustic features of a particular call, the phee, reveal more distinct differences between the groups.

Strengths:

The authors have identified that ACC lesions in early life have no or little influence on certain aspects of vocal behavior (e.g. call rate, call intervals) but larger impacts on other aspects (e.g. acoustic features of phee calls). This is difficult data to collect, especially in the difficulties of that particular time period. This data is a valuable addition to the literature on the effects of the ACC on vocal production and sparks intriguing follow-up questions on the role of different acoustic features (as related to emotional content) on vocal interactions with conspecifics over the lifespan.

The histological methods and resulting quantification of neural changes in the lesioned area and in downstream areas of interest are intriguing given the large time gap between the lesion and these analyses.

The changes to the text, figures, and additional supplemental figures to my previous review requests have made it easier to determine if conclusions are supported by the data in the manuscript. Examples include the quantification of the loss of neurons and increase in glial cells, the inclusion of changes in body weight and grip strength that could also be a result from the lesions affecting vocal behavior, and additional details on analysis methods.

Weaknesses:

The article emphasizes vocal social behavior. However, marmoset infants are recorded in isolation which allows for examining the development of vocal behavior in that particular context - reaching out to conspecifics. The text now covers the relationship between 'social' information in calls and development in this particular context. However, early-life maturation of vocal behavior is strongly influenced by social interactions with conspecifics. For example, the transition of cries and subharmonic phee calls which are high-entropy calls to more low-entropy mature phee calls is affected by social reinforcement from the parents. And this effect extends cross-context, where differences in these interaction patterns extend to vocal behavior when the marmosets are alone. Together, the results are interesting and important but may not fully capture the changes resulting from direct social interactions.

Additionally, it is an intriguing finding that the infants' phee calls have acoustic differences being 'blunted of variation, less diverse and more regular'. Though the text about how the social message conveyed by these infants was 'deficient, limited, and/or indiscriminate' is now better explained with additional text from human studies, it is still an assumption that this would directly translate to marmoset communication. Thus, experiments directed at the responses of other marmosets to these calls would still be important.

Reviewer #2 (Public review):

Summary:

Nagarajan et al. investigate the role of the anterior cingulate cortex (ACC) in vocal development of infant marmoset monkeys using lesions in this brain area. Many previous studies show that ACC plays an important role in volitional and emotion-driven vocal behavior in mammals. The experiments Nagarajan et al. performed strengthen the long-standing hypothesis that ACC influences the development of social-vocal behavior in non-human primates. Furthermore, their anatomical studies support the idea of cortical structures exerting cognitive control over subcortical networks for innate vocalization, and thus, enabling mammals to perform flexible social-vocal communication.

Strengths:

Many invasive behavioral studies in monkeys often use 2-3 animals. The authors used a sufficiently high number of animals for their experiments. This increases the power of their conclusions.

The study also investigates the impact of ACC lesions on downstream areas important for innate vocal production. This adds further evidence to the role of ACC on influencing these subcortical regions during vocal development and vocal behavior in general.

Weaknesses:

The study only provides data up to the 6th week after birth. Given the plasticity of the cortex, it would be interesting to see if these impairments in vocal behavior persist throughout adulthood or if the lesioned marmosets will recover their social-vocal behavior compared to the control animals. The authors give a reasonable explanation for why they did not provide this data.

Even though this study focuses entirely on the development of social vocalizations, providing data about altered social non-vocal behaviors that accompany ACC lesions is missing. This data can provide further insights and generate new hypothesis about the exact role of ACC in social-vocal development. For example, do these marmosets behave differently towards their conspecifics or family members and vice versa, and is this an alternate cause for the observed changes in social-vocal development? Unfortunately, the authors are unable to provide that data. Hopefully, this will be the goal of future studies.

<https://doi.org/10.7554/eLife.97125.2.sa2>

Reviewer #3 (Public review):

Summary:

In this manuscript, Nagarajan et al. study the impact of early damage to the anterior cingulate cortex (ACC) on the vocal development of marmoset monkeys. AAC lesions were performed on neonatal marmosets and their vocal patterns and the spectrotemporal features of their calls were analyzed compared to control groups during the first six weeks of life. While the vocal repertoire was not significantly affected by ACC lesions, the authors described notable differences in the social contact call, the phee call. Marmosets with ACC damage made fewer social contact calls, and when they did, these calls were shorter, louder, and monotonic. Additionally, the study revealed that ACC damage in infancy led to

permanent alterations in downstream brain areas involved in social vocalizations, such as the amygdala and periaqueductal gray.

Strengths:

This study suggests that the ACC plays a crucial role in the normal development of social vocal behavior in infant marmosets. Studying vocal behavior in marmosets can provide insights into the neural mechanisms underlying human speech and communication disorders due to their similarity in brain structure and social behavior.

The methods are robust and reliable with precise localization of the lesions with neuroimaging and histological examination.

<https://doi.org/10.7554/eLife.97125.2.sa1>

Author response:

The following is the authors' response to the original reviews

Reviewer #1 (Public Review):

The article emphasizes vocal social behavior but none of the experiments involve a social element. Marmosets are recorded in isolation which could be sufficient for examining the development of vocal behavior in that particular context. However, the early-life maturation of vocal behavior is strongly influenced by social interactions with conspecifics. For example, the transition of cries and subharmonic phee which are high-entropy calls to more low-entropy mature phee is affected by social reinforcement from the parents. And this effect extends cross context where differences in these interaction patterns extend to vocal behavior when the marmosets are alone. From the chord diagrams, cries still consist of a significant proportion of call types in lesioned animals. Additionally, though it is an intriguing finding that the infants' phee calls have acoustic differences being 'blunted of variation, less diverse and more regular,' the suggestion that the social message conveyed by these infants was 'deficient, limited, and/or indiscriminate' is not but can be tested with, for example, playback experiments.

We recognize that our definition of vocal social behavior is not within the normal realm of direct social interactions. We were particularly interested in marmoset vocalizations as a social signal, such as phee, cries and twitter, even when their family members or conspecifics are not visibly present. Generally speaking, in the laboratory, infant marmosets make few calls when in the presence of another conspecific, but when isolated they naturally make phee calls to reach out to their distantly located relatives. In this context, while we did not assess the animals interacting directly, we assessed what are normally referred to as 'social contact calls,' hence the term 'social vocalizations.' Playback recordings might provide potential evidence of antiphonal calling as a means of social interaction and might reveal the poor quality of the social message conveyed by the infant, but even here, the vocalizing marmoset would be calling to a non-visible conspecific. Thus, although our experiment lacked a direct social element, our data suggest that in the absence of a functioning ACC in early life, infant calls that convey social information, and which would elicit feedback from parents and other family members, may be compromised, and this could potentially influence how that infant develops its social interactive skills. We have now commented on the significance of social vocalizations in the introductory text (page 3) and discussion (page 15).

The manuscript would benefit from the addition of more details to be able to better determine if the conclusions are well supported by the data. Understanding that this is

very difficult data to get, the number of marmosets and some variability in the collection of the data would allow for the plotting of each individual across figures. For example, in the behavioral figures, which is the marmoset that is in the behavioral data that has a sparing of the ACC lesion in one hemisphere? Certain figures, described below in the recommendations for the authors, could also do with additional description.

Thanks for these suggestions. We have plotted the individual animals in the relevant figures and addressed the comments and recommendations listed below.

Reviewer #1 (Recommendations For The Authors):

Given the number of marmosets, variability in the collected data, lesion extent, and different controls, I would like to see more plots with individuals indicated (perhaps with different symbols). More details could also be added for several plots.

Figure 2D (new) and 2E now have plots that represent the individual animals, each represented by a different symbol.

Figure 2A) Since lesions are bilateral, could you also show the extent of the lesions on the other side for completeness?

Our intention was to process one hemisphere of each brain for Golgi staining to examine changes in cell morphology in the ACC and associated brain regions following the lesion. Unfortunately, the Golgi stain was unsuccessful. Consequently, we were unable to use the tissue to reconstruct the bilateral extent of the lesion. We did, however, first establish the bilateral nature of the lesion through coronal slices of the animals MRI scan before processing the intact hemisphere to confirm the bilateral extent of the lesion. The MRI scans (every 5th section) for each control and lesioned animal is compiled in a figure in the supplementary materials (Fig. S1). These scans show that the ACC-lesioned animals have bilateral lesions with one animal (ACC1) showing some sparing in one hemisphere, as we noted in the text. We have now made reference to this supplemental figure in the text (page 5).

Figure 2B/C) In Figure 2B, control and ACC lesions are in the columns while right next to it in 2C, ACC lesion and control are in the rows. Could these figures be adjusted so that they are consistent?

We have now adjusted these figures and updated the figure legends accordingly.

Figure 2C) Is there quantification of the 'loss of neurons and respective increase in glial cells at the lesioned site especially at the interface between gray and white matter'? There are multiple slices for each animal.

Thanks for suggesting this. We have now quantified these data which are presented as a new graph as Fig. 2D. These data revealed a significant loss of neurons (NeuN) in the ACC group as well as an increase in glial cells (GFAP and Iba1) relative to the controls. The figure legend and results have also been updated.

Figure 2C) It is difficult for me to distinguish between white and purple - could you show color channels independently since images were split into separate channels for each fluorophore?

Fig. 2C has been revised to better visualize the neurons and glia at the gray and white matter interface. We found that grayscale images for each channel offered a better contrast than

separating the channels for each fluorophore.

Figure 2C/D) I like how there are individual dots here for the individual marmosets. Since there are four in each group, could they be represented throughout with symbols (with a key indicating the pair and also the control condition)? For example, were there changes in the histology for control animals that got saline injections as opposed to those that didn't get any surgery?

We have highlighted the individual animals with different symbols in the figures. Although some animals were twin pairs, it was not possible to have twins in all cases. Only two sets were twins. We have indicated the symbols that represent the twin pair in Fig. 2 as well as the MRI scans of the twin pairs in Fig. S1. There were no observed changes in histology for the sham animals relative to the other non-sham controls. The MRI scan for one sham CON2 shows herniated tissue in the right hemisphere which is a normal consequence of brain exposure caused by a craniotomy.

Figure 3D-E) Here, individual data points could be informative especially given that some animals are missing data past the third week.

To prevent cluttering the figure with too many data points, we have added the sample size for each group in the figure legend (pages 33).

Figure 3D/F) What exactly is the period that goes into this analysis? In the text, 'Further analysis showed that the ACC lesion had minimal effects on the rate of most call types during this period'. Is this period from weeks 3 to 6 relative to the proportions in week 2? I think I also don't quite understand the chord diagram. The legend says 'the numbers around each chord diagram represents relative probability value for each call type transition' so how does that relate to the proportion of these call types? It looks like there is a wider slice for cries for ACC-lesioned animals each week. I also don't see in the week 4 chord diagram, the text description of 'elevation in the rate of 'other' calls, which comprised tsik, egg, eck, chatter and seep calls. These calls were significantly elevated in animals after the ACC lesion.'

We apologize for the confusion. Fig 3D and Fig 3F are not directly related. Fig. 3D shows the different types of emitted calls. The figure shows the averaged data per group pooled from post-surgery weeks (week 3 – week 6). It represents the proportion of individual call types relative to the total number of calls during each recording period. The only major finding here was the increased rate of 'other' calls comprising tsik, egg, ock, chatter and seep calls. These calls were significantly elevated in animals after the ACC lesion.

While Fig. 3D represents the differences in the proportion of calls, the chord diagrams in Fig. 3F represents the probability of call-to-call transition obtained from a probability matrix. At postnatal week 6, marmosets with ACC lesions showed a higher likelihood of transitions between all call types, but less frequent transitions between social contact calls relative to sham controls. The chord diagrams visualize the weighted probabilities and directionality of these transitions between the different call types. Weighted probabilities were used to account for variations in call counts. The thickness of the arrows or links indicates the probability of a call transition, while the numbers surrounding each chord diagram represent the relative probability value for each specific transition. We have now reworded the text and clarified these details in the figure legend (pages 32-33).

Figure 3E) How is the ratio on the y-axis calculated here?

The y-axis represents the averaged value of the ratios of the number of social contact calls relative to non-social contact calls in each recording per subject per group (i.e., $\bar{x}$ (# social calls / # non-social calls). This is now included in the figure legend and the axis is updated (page 32).

Also, cries could be considered a 'social contact call' since they are produced by infants to elicit responses from the parents. There is also the hypothesis in the literature that cries transition into phee.

The reviewer is correct. Cries are often considered a social contact call because they elicit parental feedback. We decided to separate cry-calls from other social contact calls for two reasons. First, in our sample, we found cry behavior to be highly variable across the animals. For example, one control infant cried incessantly whereas another control infant cried less than normal. This extreme variability in animals of the same group masked the features between animals that reliably differentiated between them. Second, cry-calls elicit feedback from parents who are normally within the vicinity of the infant whereas phee calls elicit antiphonal phee calls from any distantly located conspecific. In other words, the context in which these calls are often elicited are very different.

The use of 'syntactical' is a bit jarring to me because outside of linguistics, its use in animal communication generally refers to meaning-bearing units that can be combined into well-formed complexes such as pod-specific whale songs or predator alarm calls with concatenated syllable types in some species of monkeys. To my knowledge, individual phee syllables have not been currently shown to carry information on their own and may be better described as 'sequential' rather than 'syntactical'.

We agree. We have made this change accordingly.

Figure 4B) How many phee calls with differing numbers of syllables are present each week? How equal is the distribution given that later analyses go up to 5 syllables?

The total number of phee calls with differing number of syllables ranged between 20-40 phees. This number varied between subjects, per week. The most common were 3- and 4-syllable phee calls which ranged from 7-15. Due to this variability, Fig. 4B presents the average syllable count. The axis is now updated.

Figure 4C-E) How is the data combined here? Is there a 2nd syllable, the combined data from the 2nd syllable from phee calls of all lengths (1 - 5?). If so, are there differences based on how long the total sequence is?

The combined data represents the specific syllable (e.g., the 1st syllable in a 2-syllable phee, in a 3-syllable phee and in a 4-syllable phee) irrespective of the length of the sequence in a sequence. No differences were observed between 2nd syllable in a 2 syllable phee and 2nd syllable in a 3 or a 4 syllable phee. We have included this detail in the figure legend (page 33-34).

So duration is a vocal parameter that is highly dependent on physical factors such as body size and lung volume, where there differences in physical growth between the pairs of ACC-lesioned marmosets and their twins? Entropy is less closely tied to these physical factors but has previously been shown to decrease as phee calls mature, which we can also see in the negative relationship of the control animals. Do you know of experiments that show that lower entropy calls are more 'blunted'?

Thank you for raising the important issue of physical growth factors. For twin pairs, it is not uncommon for one infant to be slightly bigger, heavier or stronger than the other presumably because one gets more access to food. With increasing age, we did not observe significant changes in bodyweight between the groups. We examined grip strength in all infants as a means of assessing how well the infant was able to access food during nursing. Poor grip strength would indicate a lower propensity to ‘hang on’ to the mother for nursing which could lead to lower weight gain and reduced physical growth. We found that both grip strength and body weight increased as the infants got older and both parameters were equivalent. We have included an additional figure to show the normal increase in both weight and grip strength to the supplemental materials (Fig. S3) and have made reference to this in the text (page 8).

As for entropy, its impact on the emotional quality of vocalizations has not been systematically explored. Generally speaking, high entropy relates to high randomness and distortion in the signal. Accordingly, one view posits low-entropy phee calls represent mature sounding calls relative to noisy and immature high-entropy calls (e.g., Takahasi et al 2017). In the current study, the reduction in syllable entropy observed for both groups of animals with increasing age is consistent with this view. At the same time entropy can relate to vocal complexity; high entropy refers to complex and variable sound patterns whereas low entropy sounds are predictable, less diverse and simple vocal sequences (Kershenbaum, A. 2013. Entropy rate as a measure of animal vocal complexity. *Bioacoustics*, 23(3), 195–208). One possibility is that call maturity does not equate directly to emotional quality. In other words, a low-entropy mature call can also be lacking in emotion as observed in humans with ACC damage; these patients show mature speech, but they lack the variations in rhythms, patterns and intonation (i.e., prosody) that would normally convey emotional salience and meaning. Our observation of a reduction in phee syllable entropy in the ACC group in the context of being short and loud with reduced peak frequency is consistent with this view. Our use of the word ‘blunt’ was to convey how the calls exhibited by the ACC group were potentially lacking emotional meaning. Beyond this speculation, we are not aware of any papers that have examined the relationship between entropy and blunted calls directly. We have now included this speculation in the discussion (pages 12-13).

Reviewer #2 (Public Review):

The authors state that the integrity of white matter tracts at the injection site was impacted but do not show data.

We have added representative micrographs of a control and ACC-lesioned animal in a new supplementary figure which shows the neurotoxin impacted the integrity of white matter tracts local to the site of the lesion (Fig. S2).

The study only provides data up to the 6th week after birth. Given the plasticity of the cortex, it would be interesting to see if these impairments in vocal behavior persist throughout adulthood or if the lesioned marmosets will recover their social-vocal behavior compared to the control animals.

We agree. Our original intention was to examine behavior into adulthood. Unfortunately, the COVID-19 pandemic compromised the continuation of the study. We were limited by the data that we were allowed to acquire due to imposed restrictions. Some non-vocalization data collected when the animals were young adults is currently being prepared for another paper.

Even though this study focuses entirely on the development of social vocalizations, providing data about altered social non-vocal behaviors that accompany ACC lesions is missing. This data can provide further insights and generate new hypotheses about the

exact role of ACC in social vocal development. For example, do these marmosets behave differently towards their conspecifics or family members and vice versa, and is this an alternate cause for the observed changes in social-vocal development?

We agree. At the time however, apparatus for assessing behavior between the infant's family and non-family members was not available. Assessing such behaviors in the animals holding room posed some difficulty since marmosets are easily distracted by other animals as well as the presence of an experimenter, amongst other things. This is an area of investigation we are currently pursuing.

Reviewer #3 (Public Review):

It is striking to find that the vocal repertoire of infant marmosets was not significantly affected by ACC lesions. During development, the neural circuits are still maturing and the role of different brain regions may evolve over time. While the ACC likely contributes to vocalizations across the lifespan, its relative importance may vary depending on the developmental stage. In neonates, vocalizations may be more reflexive or driven by physiological needs. At this stage, the ACC may play a role in basic socioemotional regulation but may not be as critical for vocal production. Since the animals lived for two years, further analysis might be helpful to elucidate the precise role of ACC in the vocal behavior of marmosets.

Figure 3D. According to the Introduction "...infant ACC lesions abolish the characteristic cries that infants normally issue when separated from its mother". Are the present results in marmosets showing the opposite effect? Please discuss.

To date, the work of Maclean (1985) is the only publication that describes the effect of early cingulate ablation on the spontaneous production of 'separation calls' largely construed as cries, coos and whimpers in response to maternal separation. All of this work was largely performed in rhesus macaques or squirrel monkeys. In addition to ablating the cingulate cortex, Maclean found that it was necessary to ablate the subcallosal (areas 25) and preseptal cingulate cortex (presumably referring to prelimbic area 32) to permanently eliminate the spontaneous production of separation cry calls. Our ablation of the ACC was more circumscribed to area 24 and is therefore consistent with MacLean's earlier work that removal of ACC alone does not eliminate cry behavior. In adults, ACC ablation is insufficient at eliminating vocalization as well. We make reference to this on pages 13-14 of the discussion.

Figure 3E and Discussion. Phees are mature contact calls and cries immature contact calls (Zhang et al, 2019, Nat Commun). Therefore, I would rather say that the proportion of immature (cries) contact calls increases vs the mature (phee, trill, twitters) contact calls in the ACC group. Cries are also "isolated-induced contact calls" to attract the attention of the caregivers.

The reviewer is correct in that cries are directed towards caregivers but in our sample, cry behavior was highly variable between the infants. Consequently, in Fig. 3E social contact calls include phee, twitter and trill calls but does not include cries which were separated (see also response to reviewer #1). Many of the calls made during babbling were immature in their spectral pattern (compare phee calls between Fig. 3A and 3B). Cries typically transitioned into phees, twitters or trills before they fully matured. Fig 3E shows that the controls made more isolation-induced social contact calls at postnatal week 6 which were presumably maturing at this time point. Thus, if anything, there was an increase in the proportion of mature contact calls vs immature contact calls with increasing age.

Figure 4D. Animal location and head direction within the recording incubator can have significant effects on the perceived amplitude of a call. Were these factors taken into account?

The reviewer makes an excellent observation. Unfortunately, we did not account for location and head direction because the infants were quite mobile in the incubator. The directional microphone was hidden from view because the infants were distracted by it, and positioned ~12 cm from the marmoset, and placed in the exact same location for every recording. In addition, calls with phantom frequencies were eliminated during visual inspection of spectrograms. Beyond these details, location and head direction were not taken into account.

Figure 4E. When a phee call has a higher amplitude, as is the case for the ACC group (Figure 4D), the energy of the signal will be concentrated more strongly at the phee call frequency ~8KHz. This concentration of the energy reduces the variability in the frequency distribution, leading to lower entropy. The interpretation of the results should be reconsidered. A faint call (control group) can exhibit more variability in the frequency content since the energy is distributed across a wider range of frequencies contributing to higher entropy. It can still be "fixed, regular, and stereotyped" if the behavior is consistent or predictable with little variation. Also, to define ACC calls as "monotonic" I would rather search for the lack of frequency modulation, amplitude variation, or narrower bandwidth.

We very much appreciate this explanation. We were able to identify the maximum frequency that closely matched pitch of a sound for each syllable in a multisyllabic phee. New Fig. 4E shows that the peak frequency for each phee syllable was lower in the ACC-lesioned monkeys which may directly translate to the low entropy observed in this group. The term "monotonic" was used to relate our data to the classical and long-standing evidence of human ACC lesions causing monotonous intonation of speech. When all factors are taken into account, it is evident that the vocal phee signature of the ACC-lesioned animal was structurally different to the controls implicating a less complex and stereotyped ACC signal. Further studies are needed to systematically explore the relationship between entropy and emotional quality of vocalizations

Apart from the changes in the vocal behavior, did the AAC lesions manifest in any other observable cognitive, emotional, or social behavior? ACC plays a role in processing pain and modulating pain perception. Could that be the reason for the observed increase in the proportion of cries in the ACC group and the increase in the phee call amplitude? Did the cries in the ACC group also display a higher amplitude than the cries in the control group?

It was our intention to acquire as much data as possible from these infants as they matured from a cognitive, social and emotional perspective. Unfortunately, our study was hampered by variety of reasons including the COVID-19 pandemic which imposed major restrictions on our ability to continue with the experiment in a time sensitive manner. In addition, the development and construction of the custom apparatus to measure these behaviors was stalled during this period further preventing us from collecting behavioral data at regular time intervals. As for the cry behavior, the number of cries, in the ACC group were very low especially at postnatal week 5 and 6. Consequently, there were very few data points to work with.

Discussion. Louder calls have the potential to travel longer distances compared to fainter calls, possess higher energy levels, and can propagate through the environment more

effectively. If the ACC group produced louder phee syllables, how could be the message conveyed over long distances "deficient, limited, and/or indiscriminate"?

Thanks for raising this interesting concept. Not all calls emitted by the animals were loud. We specifically examined the long-distance phee call in this regard. The phee syllables emitted by the ACC group were high amplitude with low frequencies, short duration and low entropy. Taking these factors into account, it is conceivable that the phee calls produced by the ACC group could not effectively convey their message over long distances despite their propagation through the environment. We have made reference to this in the discussion where we focus is specifically on the phee calls only (pages 12).

Abstract: Do marmosets have syntax? Consider replacing "syntactical" with a more appropriate term (maybe "syntax-like").

Thanks for this suggestion. We have replaced the term syntactical with 'sequential' as per the recommendation of reviewer #1.

Introduction: "...cries that infants normally issue when separated from its mother". Please replace "its" with "their".

This has been corrected.

Results: Is the reference to Fig 1B related to the text?

We have included and referred to Fig. 1B in the text (results and methods) to show other researchers how they can use this technique as a reliable and safe means of monitoring tidal volume under anesthesia in small infant marmoset without intubation.

I understand that both "spectrograph" and "spectrogram" are used to analyze the frequency content of a signal. Nevertheless, "spectrogram" refers to the visual representation of the frequency content of a signal over time, and this term is commonly used in audio signal processing and specifically in the vocal communication field. I would recommend replacing "spectrograph" with "spectrogram".

Thanks for this suggestion. We have corrected this throughout the manuscript.

(Concerning the previous comment in the public review). Cries are uttered to attract the attention of the caregivers. The increase in the proportion of cries in the ACC group does not match the sentence: "...these infants appeared to make little effort in using vocalizations to solicit social contact when socially isolated".

We apologize for the confusion. It is not the case that the ACC animals make more cries. Cry calls were highly variable amongst the animals. Consequently, although Fig 3D gives the impression that the proportion of cries is higher in ACC animals they did not differ significantly from the controls. Due to their high variability, cries were removed in the measurement of social contact. Accordingly, Fig. 3E does not include cry behavior; it shows that the ACC animals engage less in social contact calls.

Related to Figure 3. What is the difference between "egg" and "eck" calls? Do you mean "ock"?

We apologize. This was a typo. It should be ock calls.

Figure 4B. Is the sample size five animals per group and per week? Overlapping data points seem to be placed next to each other. Why in some groups (e.g. ACC 6 weeks) less than five dots are visible?

The sample size differed per week because of the lack of recording during the COVID restrictions. In Fig 4b, we have now separated the overlapping dots. We have also added the sample size of the groups in the figure legends.

Would the authors expect to see stronger differences between the lesioned and the control groups when comparing a later developmental stage? The animals were euthanized at the age of

These speculation is certainly feasible and yes, we were hoping to establish this level of detail with testing at later developmental stages. This is an aspect of development we are currently pursuing.

Could these experiments be conducted?

I'm afraid these animals are longer available, but we are currently conducting experiments in other animals with early life neurochemical manipulations who show behavioral changes into early adulthood.

ACC lesion: It is reported that the lesions extended past 24b into motor area 6M. Did the animal display any motor control disability?

Surprisingly, despite the lesion encroaching into 6M, these animals showed no observable motor impairment. We assessed the animals grip strength and body weight and discovered normal strength and growth in weight in both controls and the lesioned group. We have added this data as supplemental information (Fig. S3).

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