We thank the reviewers for carefully evaluating this manuscript and providing constructive feedback. Below, we provide a detailed response to each of the concerns raised and outline the areas in the text that have been revised.

**ESSENTIAL REVISIONS**  
  
**1. Whether one or both sexes were studied is not mentioned in the abstract. Please make sure to update the abstract in the article file and on the submission form.**  
  
The abstract and submission form have been updated.

**Reviewer 1:**

**In this study, the authors deepen the understanding of the mechanisms that drives spontaneous activity in the developing cochlea. The authors draw upon the many in vitro imaging and whole cell recording and in vivo imaging techniques the lab has established to characterize the spontaneous activity in the developing auditory system and to reveal the mechanisms that link spontaneous release of ATP from inner supporting cells (ISCs) to spontaneous depolarization of inner hair cells, which in turn synaptically activate spiral ganglion cells and drive co-activation of isofrequency bands in the inferior colliculus. In particular they take advantage of a specific antagonist of the P2RY1 receptor (MRS2500) which they recently characterized as an effective blocker of cochlea activity in P6 mice and rats (Babola at el Elife 2020). In the current study, the authors show that the primary mechanisms that mediate spontaneous activity postnatally -- activation of P2RY1 receptors on ISCs - initiate the activity throughout the period of development prior to the onset of hearing. They identify interesting changes across development including the age at which when the spontaneous ATP-mediated inward currents in ISCs first appear (E14 in the more mature basal region of cochlea while absent at this age in the apex), and that crenations do not appear until a few days after the ATP autocrine signaling is observed. They also add to the growing (albeit confusing - see below) impact of nicotinic acetylcholine receptor signaling on modulation of this activity via efferent signals they focused on earlier ages.**

Thank you for appreciating the novelty and interest of these studies.  
  
**2. Embryonic dissections are not described in the methods**

Information regarding embryonic dissections is now included in the methods.  
  
**3. Figure 4 - the authors show that the crenations do not appear until P7 despite the presence of ATP-induced inward currents prior to that. For these studies the authors focused on the apical region of the cochlea which is less mature than the basal. If they do have basal data that would be great to make note of any differences. If these experiments were done exclusively in the segments taken from the apex, then they should be explicit about that in the figure caption. In fact this should be noted in ALL of the figures whether recording are done in apex or base of cochlea - this was difficult to keep track of throughout the paper.**

We have now included electrical recordings (Figure 1 and Figure 3) and crenation imaging of ISCs (Figure 4) in the basal portions of cochlea. We found XXX. Text clarifying recording locations was inserted throughout the figure legends and text.  
  
**4. In the description of the results shown in Figure 6 the authors assert that the presence of phalangeal cells contributes to muted response in apex - the authors need to better explain why this is the case.**

Additional detail is now included near the end of the results section “Correlated activation of IHCs requires activation of ISC P2Y1 receptors.” Because extrusion of K+ into the extracellular space requires Ca2+-dependent activation of TMEM16A (Wang et al., 2015), lack of Ca2+ transients in the apical inner phalangeal cells suggests that K+ extrusion is limited near the IHCs, which is consistent with the observation that IHCs in the apex are not activated as those in the middle in apex for a given ATP-release event.   
  
**5. Figure 10 - the authors show that after P7, MRS2500 does not impact activity in the inferior colliculus (Babola 2020). Their control for "CNS effects" is that they observe no impact on retinal waves. The authors need to better explain why this is a reasonable control since ATP signaling does not impact retinal waves.**

We have clarified the language in the results section “P2RY1 activity is required for spontaneous activity *in vivo* at P1”. While the blood-brain-barrier has formed by this developmental stage (Ben-Zvi et al., 2014), we do not know how the BBB permeability of MRS2500 and it is possible that MR2500 may be antagonizing P2RY1 within the CNS. In particular, functional P2RY1 has been reported in astrocytes, where activation of P2RY1 enhances K+ uptake and decreases neuronal excitability (Wang et al., 2012). Because neuronal activity was reduced within the auditory system and unchanged in the SC (Figure 10), this suggests that the compound is specifically acting along the auditory axis. Moreover, the previous results suggest that blocking astrocytic P2RY1 would result in enhanced, rather than diminished activity.  
  
**6. The authors use two mouse lines - an alpha9-nAChR KOP and a GOF of mouse to attempt to gain insight into the role of efferent signaling in initiating/modulating these events (Figure 11). It felt a little bit that these are measurements that are coming from left field given the cohesiveness of the rest of the paper. I leave it to authors to consider whether they want to keep it in this study. I do think this is an important piece of data to publish, despite it being confusing, and for the most part the authors have been quite cautious in their interpretation. I think the authors should more explicitly consider the possibility of developmental compensation, particularly since they themselves showed homeostatic regulation of spontaneous activity. In particular, the authors may want to consider testing to see if the spontaneous activity observed in nAChR KO mouse is blocked by MRS2000. To be very clear, I do not think this experiment needs to be done for this study to be published - it is just a suggestion.**

We have revised the results to bridge into alpha9 experiments more cohesively. Our primary purpose aim was to determine if efferent signaling was responsible for generating bursts of action potentials *in vivo*, as this has been a proposed mechanism for generating spontaneous activity in the developing cochlea (Johnson et al., 2011). Because calcium transients persist in the alpha9 KO at the same frequency as WTs, it suggests that the initiation machinery is unaffected. We agree that it is not definitive and that constitutive knockout/knockin of alpha9 protein could result in circuit-level changes, however, coupled with the fact that acute cochleae, in which the efferents have been severed, exhibit prominent bursting activity and the current studies demonstration that P2ry1 inhibition profoundly decreases transients in the IC, these experiments provide additional evidence that efferents do not initiate burst firing in the cochlea.  
  
**7. There are a few small problems with figures**  
 **7a. Figure 1 -- The figure captions are A-E but the figure only has A-D.**   
**7b. There are a few examples in figures where various parts of figure (e.g. white box in Figure 3, asterisks in Figure 6) are described in main text but they should be described in the figure caption.  
7c. Caption in Figure 8 should include mouse line.**

Captions have been corrected and updated with clarifying information

**7d. Figure 9 - more detail is needed for panels A and B.**   
  
More detail has been added in the figure captions and orientation indicators have been added to the panel. *Maybe we should rework the panel to show our imaging setup… i.e. like Fig 1 from the 2018 paper*

**Reviewer 2:**  
  
**This paper reports high quality data describing the onset of spontaneous activity in the developing auditory system and the effects of purinergic and cholinergic signaling on this activity. The authors previously showed that P2ry1 is necessary for spontaneous activity in the developing cochlea. Here, they investigate whether spontaneous activity is driven by P2ry1-mediated signaling throughout development or whether other mechanisms come into play at other stages, as is the case in the visual system where early retinal waves are dependent on gap junctions but spontaneous activities at later stages are dependent on cholinergic and glutamatergic signaling. To address these questions, they use a combination of electrophysiological recordings and calcium imaging, both in excised cochlea and in the inferior colliculus (IC) of awake animals, using well-characterized pharmacological agents to block P2ry1 and/or glutamate receptors and two mouse mutants to alter cholinergic signaling. The primary advance over previous work is that the experiments are performed at embryonic timepoints, together with several postnatal stages. The data show that spontaneous activity initiates just before birth in a base-to-apex gradient and requires P2ry1 at all stages, with inner supporting cells inducing coordinated activity in gradually more IHCs, such that SGNs show well-coordinated bursts of activity (mediated by glutamate signaling) by P0. The data also show that ISCs can exhibit spontaneous activity without crenating, drawing attention to how changing cell properties might influence the spread of activity in the developing cochlea. Further, characterization of spontaneous activity in the IC reveals that the iso-frequency bands become narrower with development. The pattern of activity in the IC is altered when cholinergic signaling is either blocked or enhanced, but not in ways that would have been predicted from previous characterization. Altogether, these are useful descriptive data, as knowing when spontaneous activity emerges and how its temporal structure changes over time offers valuable insights into the dynamics of pre-hearing cochlear activity.**

**The authors present the work as being significant from the perspective of drawing contrast with the visual system where the mechanism driving spontaneous activity changes as an extended phase of postnatal synaptogenesis results in drastic expansion of cholinergic and glutamatergic drives in retinal circuits. This is a very interesting perspective and undoubtedly something the field stands to benefit from having been elucidated. Beyond that, the data help establish a foundation for studying the developmental origins and impact of spontaneous activity from the cochlea to the IC. The authors have done rigorous work and there are no obvious problems with the particulars of their experiments or data analysis. However, there are some problems with how the findings are placed into the context of the question as it is framed, as well as with the interpretations of some key experiments.**

We thank the reviewer for highlighting the importance of the study and the approaches used within.

**8. Previous work from this group (Babola et al., 2020) showed that P2ry1 is a key player in driving spontaneous activity in the cochlea. This was based on experiments done in P6-P8 mouse and rat pups. Note that, at these stages, glutamatergic signaling and cholinergic transmission in the cochlea are both already in place. This already shows that the mechanism underlying cochlear and retinal spontaneous activities are different. Likewise, this group also already showed that spontaneous activity persists in the absence of glutamatergic transmission, albeit with altered temporal characteristics (Sun et al., 2018). Furthermore, Clause et al., 2014 showed that spontaneous activity persists without functional cholinergic signaling within the cochlea (in alpha9 KO mice). Taken together, there is already strong evidence in the literature indicating that the mechanism underlying spontaneous activity in the auditory system is fundamentally different than those generating retinal waves. From this point of view, the data, as presented, add only incrementally to a question that has already been partially answered. This is not a problem in and of itself, as we should encourage independent replication of results. However, the paper should be re-written to more accurately reflect what was already known about the question and place these new findings into proper context.**

We apologize if the contrast we were intending to make was not clear.We have no doubts that the mechanisms responsible for generating spontaneous activity in the visual and auditory systems are independent. We intended to contrast the dynamic nature of retinal wave generation, which goes through three distinct mechanisms each producing unique patterns of neuronal activity, to the static mechanisms observed in the auditory system. Our previous studies demonstrated that electrical activity in ISCs is dependent on purinergic receptors from P0 to shortly after hearing onset (Tritsch and Bergles, 2010), however, both the onset of ISC spontaneous activity and which purinergic receptors were involved throughout this period were unknown. This study builds on these past observations by demonstrating that ISC electrical activity begins in late embryonic development and requires P2RY1 signaling throughout both embryonic and early postnatal timepoints. We have made revisions to the introduction and discussion to make this contrast more apparent.  
  
**9. There is a similar problem in the presentation of the results from the alpha9 KO and GOF animals. In these experiments, patterns of spontaneous activity in the IC were characterized to gain insights into the overall contributions of cholinergic signaling, presumably at P3 (I had a hard time figuring out the timepoint, which should also be fixed). This is a very interesting question to address, as the impact of efferents on spontaneous activity remains unclear. The observations are intriguing as they do not fit simple predictions from what has been observed by analyzing cochlear explants. However, some features of the mouse models make it difficult to arrive at any clear conclusion about the role of cholinergic modulation in shaping spontaneous activity.**

The timepoints for alpha9 experiments have been added to the figure captions and text.  
  
**11. The global, constitutive KO and GOF mouse models that were used present two confounds. First, there could be compensatory changes in the circuit in the absence of cholinergic IHC modulation, so the IC activity phenotype they report could reflect just that. See this study for example:**[**https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2816210**](https://nam02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.ncbi.nlm.nih.gov%2Fpmc%2Farticles%2FPMC2816210&data=02%7C01%7Cdbergles%40jhmi.edu%7C05f6e02c119f4063b1ad08d85dc23ef2%7C9fa4f438b1e6473b803f86f8aedf0dec%7C0%7C0%7C637362437665143875&sdata=fwXYzKqRo4RE1%2Bo9pQaxfp8vLLuqpDZQObLosQu7BBM%3D&reserved=0)**/.**

We agree that constitutive knockout/knockin of alpha9 protein could result in developmental changes in circuit properties. As you’ve pointed out, vast changes occur in the transcriptional profile of the cochlea with loss of alpha9 and our previous studies demonstrate that these nascent auditory circuits have a large capacity to change in order to generate bursts of electrical activity. While our study does not directly address these possibilities experimentally, a recent BioRxiv article examined the effect of acute inhibition and excitation of cochlear-labeled efferent fibers using DREADDs while imaging the inferior colliculus (Wang et al., 2020). Acute activation of inhibitory DREADDs in cholinergic fibers deceased the correlation between IC hemispheres (similar to alpha9 KO) and activation of excitatory DREADDs increased the correlation (similar to alpha9 GOF). These data suggest that changes in the bilateral propagation of cochlear-driven spontaneous activity is the result of acute efferent signaling, rather than compensatory circuit changes. Because this study has not yet been peer reviewed, we have revised the discussion to include the possibility that circuit level changes may be responsible for the effects observed on IC activity.

**12. Second, because the models are not tissue-specific, the phenotype cannot be attributed solely to changes in cholinergic drive within the cochlea as changes in cholinergic modulation throughout the auditory axis would affect activity observed in the IC. Had the observations been made in activity patterns in IHCs and SGNs in the cochlea, it would be possible to address the question of whether the mechanisms that generate spontaneous activity also involve cholinergic signaling more directly. Although it has long been believed that Chrna9 is not expressed in the brain and is specific to vestibular and cochlear hair cells in the auditory axis, that claim has come into question of late (**[**https://www.frontiersin.org/articles/10.3389/fncel.2017.00282/full**](https://nam02.safelinks.protection.outlook.com/?url=https%3A%2F%2Fwww.frontiersin.org%2Farticles%2F10.3389%2Ffncel.2017.00282%2Ffull&data=02%7C01%7Cdbergles%40jhmi.edu%7C05f6e02c119f4063b1ad08d85dc23ef2%7C9fa4f438b1e6473b803f86f8aedf0dec%7C0%7C0%7C637362437665153869&sdata=ceaORwQobzXvMwc5YPoFX9RmTHuSPN3omFufnOead0k%3D&reserved=0) **). Even if there is no Chrna9 expression in the relevant brain areas for this to be an issue, it is still not necessarily safe to assume that other aspects of efferent feedback system (e.g., the brainstem collaterals of the OCNs into the cochlear nucleus complex) is otherwise normal when cholinergic signaling is disrupted peripherally.**

We respectfully disagree about the cited studies proposition that alpha9 is expressed in the brain. Independent studies examining in situ hybridization of alpha9 probes (Elgoyhen et al., 1994), alpha9 BAC-transgenic reporter mice (Luo et al., 1998), and RT-PCR of alpha9 transcripts in rat brain (Morley et al., 1998) reported no detectable expression in the CNS. Similarly, a new mouse model generated by the Fuchs lab using CRISPR-mediated knockin of reporter protein into the endogenous alpha9 allele revealed no notable expression in the brain (ARO, 2020). While we do not wish to expound on the experimental problems of the cited work here, a detailed critique of the methodologies used in that study and additional refuting evidence can be found in (Morley et al., 2018).

That being said, we cannot rule out potential circuit level changes as a result of altered cochlear-driven electrical activity. In fact, we would predict that modulation of bursting activity (by altering the precise patterns of action potentials) would have a profound influence on circuit development. Indeed, disruptions in the tonotopic refinement of auditory brainstem neurons has already been reported in alpha9 KO mice (Clause et al., 2014) and altering retinal wave patterning leads to pruning deficits in the superior colliculus (Xu et al., 2011). Our data demonstrate that the bilateral representation of cochlear-driven activity changes in these mice, but how these alterations ultimately effect circuit development are unclear and require generation of new genetic tools to address, i.e. mice where alpha9 can be conditionally manipulated only during development. We have revised the discussion to address the possibility of circuit level changes as a result of altered activity.

**13. The impact of efferent signaling on spontaneous activity patterns could be addressed more directly by examining spontaneous activity patterns in the cochlea of the mutant mice. I understand that the efferents may not survive long in the cochlear explants and I am curious how this affects the authors' interpretations of the wild-type data, knowing that an important feature of the circuit may not be fully represented. Nonetheless, in order to properly interpret the in vivo findings, it would seem important to know whether there are obvious differences in the patterns of spontaneous activity in the cochlea of either mouse line, perhaps for compensatory reasons. If there are good reasons why these experiments cannot be performed, the authors should at least acknowledge the possibility that any observations in the IC could be influenced by central changes.**

We feel that experiments examining the effects of efferent activity performed in acute cochleae would be problematic primarily because the feedback circuit is severed and we would be unable to reproduce a natural efferent response. While this may indeed sound like a potential confound in wildtype mice, our previous studies comparing the pattern of burst firing in acute cochlea and in vivo units in the auditory midbrain and found that many features (interspike interval histograms, accelerating and decelerating rate of action potentials, long periods of quiescence) were remarkably similar. These data indicate that acute preps: 1) do not require intact coordination of efferent activity to initiate burst firing and 2) SGN activity in acute preps is a reasonable approximation of what propagates to the CNS. The drawback of recording SGN action potentials is that you are limited to an individual unit. As shown in Figure 6, a given ATP-release event has the potential to activate groups of nearby IHCs and, subsequently, SGNs. It is possible that with a reflexive efferent circuit in place, activation of inhibitory inputs could limit the effective number of IHCs activated per event or alter the firing patterns of IHCs. For these questions to be answered, new approaches that preserve the efferent circuitry, such as 3P in vivo imaging of cochlear hair cells in wildtype and alpha9 transgenic mice, could provide new insights into the role of this elusive circuit, however, this is outside of the scope of this study. We have revised the discussion to address the possibility of circuit level changes as a result of altered activity.