Anatomical identification of primary auditory cortex in the developing gerbil

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Anatomical identification of primary auditory cortex in the developing gerbil

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May 2011

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Biology at the City College of New York

CUNY
To my girls: Cecilia, Emilia, Matilde, Loreto.
INDEX

Acknowledgments 4
Abstract 5
Introduction 7
Objectives 10
Materials and Methods 11
Results 15
- Determination of anatomical references for A1 15
- Neurovue tracer labeled neural projections between the cortex and the thalamus.
  19
- Changes in the composition of the cortex 25
- Changes in the size of the cortex 28
- Labeling of Subplate neurons 30
Discussion 33
Conclusions 36
Future Directions 37
References 39

Figures
  Figure 1 15
  Figure 2 17
  Figure 3 18
  Figure 4 20
  Figure 5 21
  Figure 6 22
  Figure 7 24
  Figure 8 25
  Figure 9 27
  Figure 10 28
  Figure 11 29
  Figure 12 30
  Figure 13 31
  Figure 14 32
Acknowledgments

My first acknowledgment goes to my mentor, Dr. Adrián Rodríguez Contreras, not only for the chance to join into his lab, but also for the things I learned there. It was a great year.

Also, my thanks to Dr. Vibhakar Kotak who gave me the chance to collaborate in the interesting project that led to this thesis.

To my dear friend Dr. Vivek Khatri, for his help and advising during my first year in the USA.

To all my colleagues/friends in the lab, for all the help during all this Masters period, the discussions, brainstorming and friendship.

To my brother Sebastian for his computational support.

And of course, I thank my wife, for her unconditional love and support all these years.

To all of them, my most sincere appreciation and gratitude.
ABSTRACT

Cortical development is an active field of study. The gerbil provides an excellent model for research because at the moment of birth its brain is rather immature, anatomically and functionally. Furthermore, the gerbil auditory cortex is particularly amenable to investigation in that the gerbil’s onset of hearing occurs approximately after 14 days of postnatal life.

Despite these advantages of the gerbil for auditory cortex development not much is known about the anatomy of the postnatal gerbil auditory cortex. For example, where is the gerbil auditory cortex? Prior to the onset of hearing, sounds cannot be used to localize auditory cortex. Therefore, the localization of the auditory cortex with anatomical landmarks alone during development is necessary. Anatomical and physiological approaches to localize the auditory cortex are published in the literature, but evaluated only in adult gerbils, and many of them are based on the fresh brain slice preparation and not applicable in vivo.

The present study compares the anatomical references of the adult gerbil published in the literature with the anatomical references in the developing gerbil. Using specific blood vessel positions as landmarks, the position of the primary auditory cortex was estimated on fixed brains and in vivo. The lipophilic tracer Neurovue was used to confirm whether the position resulting from the anatomical analysis was consistent with the position of the auditory cortex.
The results showed that anatomical references present already in the developing gerbil are consistently related to the position of the auditory cortex and they can be reliable used as landmarks to detect the actual position of the auditory cortex.
INTRODUCTION

The gerbil is an excellent model for auditory cortical development: it is easy to breed, easy to train, and more interestingly, it is born with a brain in a rather immature state (1). Nevertheless, gerbil’s brain anatomy descriptions or atlases are not easy to find in the literature, neither adult nor young, and therefore developing auditory cortex in vivo is hard to be located with precision. One approach has been developed by Kotak et al (2), using fresh thalamo-cortical brain slices: they used direct thalamic electrical stimulation to evoke discrete responses in the auditory cortex. Another effective way to locate the primary auditory cortex (A1) is presenting an auditory stimulus to the animal and recording time-locked electrophysiological responses in the temporal cortex (3). More recent studies using dextran tracers and electrophysiological recordings have shown that location of A1 in adult gerbils is in a close relation with one branch of the inferior cerebral vein and the middle cerebral artery, which together form a conspicuous loop on the surface of the brain (4). Recently, using gerbil CT brain scan images, a 3D atlas fully compatible was constructed. Unfortunately, this is available only for adult gerbils (5).

However, the anatomical landmarks defined for in adults may not be valid in the neonate or young gerbil due to changes in brain anatomy during development. During development, the gerbil brain (like other animals) will increase dramatically in size and most likely, the relative position of different functional areas will shift. In fact, the gerbil brain at birth is only 16% of the size of
the adult brain, and its growth is not a uniform process (1). Therefore, any attempt to transfer coordinates or anatomical relations may lead to erroneous conclusions for the localization of a neural structure.

Another key difference between adult and developing gerbils is the ability of hearing by itself: the young gerbil shows a functional hearing around postnatal days 14 to 15 after birth, and before that time it is deaf, and during a transitional period, (about postnatal days 11 to 13), the animal presents some auditory responses at very high sound intensities (6). This is the reason that prevents using a technique such as presenting an auditory stimulus to the animal, mentioned above, and registers the position of the time-locked response to identify A1.

In the microscopic anatomy, an important feature is evident in the organization of the cortex. The mature cortex shows a six-layered organization. Every layer receives different inputs and the main target for thalamo-cortical fibers is the layer IV (7). In the developing brain, the six cortical layers are not completely developed yet, and the thalamic connection to the cortex is not direct: thalamic fibers influence the cortex via a developmentally transient structure, the subplate layer. The subplate layer is formed by a heterogeneous population of neurons (8), located under the developing cortical plate (9). Subplate cells have an important role in leading axonal projections from and towards the cortex, the development of columnar architecture and plasticity (8,10). Later in development, the subplate cells reduce their number and only a 10% or 20% of them survive, and remain dispersed in the white matter (11) and some other
authors have used the term of layer VII to indicate this population of surviving cells (12).

The cortico-thalamic pathways have been described previously in several studies (13, 14, 8) and it is well established that the thalamic source of those connections is the medial geniculate body (MGB). The use of tracers to label neuronal tracts is a well-documented technique to study the connections between A1 and the MGB (15, 4 and 16). Combinations of the anatomical references mentioned above with the use of the modern tracer techniques can be used as a good approach to provide an excellent estimation of the position of A1 in developing gerbils, and eventually describe its development.
OBJECTIVES

To define the location of primary auditory cortex in neonate and young gerbils, prior to the onset of hearing.

To evaluate the utility of anatomical references to determine the location of primary auditory cortex in developing gerbils.

To describe the changes in thickness of the primary auditory cortex during development, the developmental changes of cortical layers and the presence of the subplate cell population.
MATERIALS AND METHODS

The studies were performed in gerbils (*Meriones unguiculatus*) ranging in the ages from 3 to 14 days of postnatal life. As a convention, the day of birth is considered P0. All these ages are previous to the onset of hearing and P14 is close to that event (6). All animals were treated according to CCNY guidelines of animal care.

The animals were overdosed with Ketamine and transcardially perfused with a solution of 0.1 M phosphate buffer (PB) and fixative solution of 4% paraformaldehyde (fix solution) and later submerged in a solution of 30% sucrose in the same fixative solution as a general procedure.

To test whether the anatomical references in the adult gerbil were present during development, we evaluated how the anatomical landmarks described in the literature (4) are related to A1 in neonate and juvenile gerbils. Using a geometric concept to locate the center of a figure, single lines were projected between the opposite sides of the area limited by the mid-cerebral artery and the inferior cerebral vein, and established that the intersection of those lines (center of the area described by the blood vessels) matched the position of A1 in the adult gerbil (Figure 1A). Next, the blood vessels in the developing gerbil brain were highlighted. Two P9 gerbils were transcardially perfused with PB solution and regular ink. The ink perfusion highlighted the blood vessels with a ubiquitous black color over the gray cortical tissue, unambiguously identifying the blood vessels. The brains were photographed in order to compare the results with
those of Büdinger and Scheich (2009; Figure 1B). These brains were additionally used to evaluate the transportation time required for the tracer to exit the cortex and reach the MGB in the later experiments.

Once the anatomical references were confirmed in the perfused brain at P9, three neonate gerbils (at ages P3, P4 and P5, one animal per ages) were anesthetized with Isoflurane 1.5% and intubated endotracheally to ensure a good alveolar concentration and anesthetic effect with the lowest possible dose of anesthesia. The scalp was removed with surgical scissors and the skull exposed. Once the preparation was ready, pictures of the surgery site were taken to compare them with the anatomical landmarks in the perfused brain and the literature (Figure 1C).

Based on the results of the ink perfusions and the anatomical references mentioned above, the gerbil’s brains were perfused using the same PB and fix solutions mentioned above. The brains were kept in the fixative solution for 3 days before any procedure. Filter paper squares coated with Neovvue tracer (0.1mm wide, 1 mm long) were placed in the expected location of A1, ~ 0.5 mm deep in the cortex. Neovvue is a lipophilic tracer excitable at 565 nm wavelength and it has been used successfully in previous studies on auditory development (17) and it is part of a new generation of lipophilic tracers which diffuse into plasma membranes in fixed tissues, eventually labeling the entire cell body and the finest axonal and dendritic branches, allowing the visualization of neuronal processes several millimeters from the point of dye insertion. The position of A1 was estimated considering the loop formed by the inferior cerebral vein and the
middle cerebral artery as the main reference, and the position of the tracer was the center of the area limited by that loop. This procedure was repeated in gerbil brains at P3, P4, P5, P12 and P14 (Figure 2).

Once the Neurovue points were placed, the brains were returned to the 30% sucrose solution and stored at room temperature for a minimum of four weeks.

After 4 weeks of tracer transport, the brains were enclosed inside a block of 10% gelatin / 30 % sucrose to facilitate the slicing and mounting. The slicing was performed in a frozen stage microtome set at 50 μm thickness using a coronal orientation. Later, the slices were mounted and analyzed under epifluorescence microscopy at 4x and 10x magnification. The images obtained were processed using Nikon NIS Elements software 3.22 for acquisition and Photoshop CS5 for later processing.

The anatomy of the slices was confirmed with the Paxino-Watson’s Rat Brain Atlas as a guide and the references (1), (4) and (15) to locate the MGB in the gerbil brain (Figure 3).

The measurements were performed in calibrated images in proportions of 2.43 μm / pixel for 4x magnification, 0.97 μm / pixel for 10x magnification and 0.25 μm / pixel for the 20x magnification from the outer layer of cortex to the deepest limit of the cortical plate, in the position adjacent to the site of placement of the Neurovue tracer for both cortical and subplate evaluations. The thickness of A1 was determined in coronal sections of the P3, P4, P5, P12 and P14 gerbils (one animal per age). For all the measurements the site of measurement was a
region of the cortical place adjacent to the tracer site. All the measurements of thickness were repeated 4 times. In order to discard any difference produced by the placement of the tracer, one set of bilateral measurements were obtained on both sides of the brain: the side of the tracer placement and the side of the intact cortex, in the same slice, and always contra lateral to the site of tracer placement.
RESULTS

Determination of anatomical references for A1

According to the Büdinger and Scheich results (4), A1 should be located in the center of the area surrounded by the mid-cerebral artery and the inferior cerebral vein (Figure 1A). After ink perfusion, the blood vessels are revealed in an outstanding black color (Figure 1B). This allowed us to detect clearly the mid cerebral artery and the inferior cerebral vein. The vasculature showed the same configuration described for the adult gerbil, in the temporal cortex.

The next step was to reveal the existence of the same references in vivo. Due to the early developmental stage, the cranial bones are actually thin and allowed a clear view of the underlying vasculature. As expected, the visualization of blood vessels was good, and it was possible to confirm the same circulatory anatomy disclosed with the ink-perfusion (Figure 1C).
Figure 1: Brain vasculature related with the auditory cortex. A, Using the image of an adult brain (modified from Büdinger and Scheich 2009) the geometric concept of “center of gravity” is applied (red lines intersection) to locate the center of the area surrounded by the mid-cerebral artery (mca) and the inferior cerebral vein (icv), which is coincident with the position of A1. B, In an ink-perfused brain the blood vessels are perfectly highlighted, and the center of gravity can be perfectly traced to detect the position of A1. C, The blood vessels are also visible in vivo, therefore it is possible to estimate the position of A1 in that condition using the same concept. OB: olfactory bulb; Cb: cerebellum; D: dorsal auditory field; DP: dorsoposterior auditory field; VP: ventroposterior auditory field; VM: ventromedial auditory field; V: ventral auditory field; AV: anteroventral auditory field; AAF: anterior auditory field; Δ: lambda suture. Scale bars in (A): 400 µm.

The P3, P4, P5, P12 and P14 brains were perfused as indicated previously, and the Neurovue were placed in the estimated position of A1 using the anatomical references mentioned above. The position of the tracer was always in located in the center of the area surrounded by the mid-cerebral artery and the inferior cerebral vein. The position of the tracer was also the most lateral point of the temporal lobe.

The vasculature used as references for this experiment were present in all the cases evaluated in this study.
Figure 2. Neurovue tracer placement. The pictures show the position of the Neurovue tracer in the perfused brains. The position was always determined under visual guidance using the anatomical references mentioned in Material and Methods and the Neurovue was inserted using a microforceps. The rulers under the brains were used to estimate the distance of tracer insertion and the occipital and frontal poles of the brain, scale is in mm. (A): P3 brain, PB and Fix perfusion. (B): P4 brain, PB and Fix perfusion. (C) P5 brain, PB and Fix perfusion. (D): P12 brain, PB and Fix perfusion. (E): P14 brain, PB and ink perfusion.

Despite the regular configuration of this circulatory landmark, its distance from the frontal pole of the brain varies as the animal grows. This is not
surprising in a developing animal. Confirming that distance of the expected position of A1 to the most anterior (rostral) point of the frontal lobe increases with time was expected, but surprisingly, the increment of distance was quite similar using the occipital edge of the cortex. The graph in Figure 3 shows that both measurements produce a similar line shape in the graph and both series present a high correlation (Pearson’s Correlation Coefficient = 0.989).

Figure 3. Neurovue distances to the frontal and occipital lobes. The graph shows the distance from the most anterior (frontal lobe, red line) and most posterior (occipital lobe, blue line) points in the cortex to the Neurovue site. Measurements were obtained in one animal at ages P3, P4, P5, P12 and P14.
Neurovue tracer labeled neural projections between the cortex and the thalamus.

The first results to be evaluated were the identification the anatomical structures required for this study, the MGB, the cortical plate and the subplate, and later the labeling properties of the Neurovue and its transport to the MGB.

The figure 4 shows the anatomical structures present in a coronal slice of a gerbil’s brain, the section corresponding to the tracer position. Based on the literature, the image obtained using the DAPI fluorescent dye allows to visualize several structures which are relevant in this study. The APTV (Anterior Pretectal Ventral nucleus) is a semicircular structure easy to identify in the image. It is not part of the auditory circuit (is part of the visual pathway) but it is a good reference to the position of the MGB, located in a more dorsal position to its lateral end in the same coronal plane. The cortical plate corresponds to the most external layers of cells in the brain, the place were the tracer was placed. Underlying that cortical plate there is a less fluorescent region, this region is defined as the subplate region. The white matter in the image corresponds to the bundle of axons connected to the infracortical structures. The nomenclature white matter may not be exact, because at these ages the myelin is not yet formed.
The position of the tracer (Figure 4, red arrow) is confirmed to be in the cortical plate, without any contact with the infracortical anatomy. Therefore, we can expect that Neurovue will be transported by cells in the auditory cortex and not by some cells or tracts from different pathways.

Figure 4. Microscopic Anatomy. Image of a P9 brain showing the main anatomical references used in this study. The red arrows show the point of
insertion of the tracer. The white arrows show the extension of the subplate region. The yellow arrow indicates the extension of the cortical plate. APTV: Anterior Pretectal nucleus ventral. MGB: Medial Geniculate Body. Magnification 4x. DAPI fluorescent dye. Scale bars: 1000 µm vertical and horizontal.

The evaluation of the Neurovue labeling was evaluated in two different ways. First, the quality of the labeling of cells and cellular processes was evaluated. Considering the lipophilic properties of the tracer, the expected result was a clear exposition of the neuron membranes and axonal tracts across the sample (Figure 5). When the Neurovue image is combined with the DAPI dye, which stains the cell nuclei, the results provide a useful image not only for tracing but also for counting cells, with blue stained nuclei surrounded by a red stained membrane and axon tracts.
Figure 5. Neurovue labeling. (A) DAPI fluorescent dye, labeling the DNA in the nuclei of the cells (yellow arrows). (B) Shows the neuronal tracts (axons) labeled by Neurovue (yellow arrows). (C) Shows the merged image Neurovue/DAPI corresponding to the same region of (A). Note the DAPI staining in the nuclei and Neurovue surrounding them (cell membrane). This is consistent with the lipophilic characteristics of the tracer. The images A and C were taken at 20x magnification at the site of labeling of a P14 brain. B is taken from a different region with better exposition of neuron axons labeling. Scale bars: A, C: 25 µm. B: 250 µm.

After two weeks of transport, the P9 brains used as evaluation showed a clear labeling from cortex towards thalamic structures under epifluorescent microscopy (Figure 6). However, the labeling in these samples did not reach the MBG. Therefore, the next experiments considered at least 4 weeks of labeling.
Figure 6. Corticothalamic labeling with Neurovue. First trial with Neurovue tracer, P9 brain. The sample was processed after 2 weeks of transport. The tracer labeled thalamocortical projection but it did not reach MGB. MGB: Medial Geniculate Body.

Once the period of 4 weeks of transport designed for the experiment was completed, the brain slices were examined under fluorescent microscopy. The images revealed the presence of a conspicuous labeling of neuronal tracts towards the thalamus, just like the previous experiments. Nevertheless, in these new samples the labeled tract was present in an extension of ~450 µm in the coronal plane and about 900 um in the horizontal plane, to finally label the MGB (Figures 7,8). The labeling in the MGB showed several magnitudes, but always detected in P3, P4, P5, P12 (not shown) and P14 animals. These results confirm that the estimation of A1 location obtained using blood vessels as a reference was correct.
Figure 7: MGB labeling by Neurovue in A1. The 3 panels show the successful labeling of the MGB transported from A1 after 4 weeks. The first column (from left to right) shows the DAPI image. The second column shows the same image using the TxRed filter to expose only the Neurovue tracer. The third column shows the first and second columns merged in one single image. The fourth column shows a zoom in of the MGB region. (A) P3 gerbil. (B) P4 gerbil. (c) P5 gerbil. Scale bar 1000 µm.
Changes in the composition of the cortex.

The six layer configuration of the cortex present in the adult gerbil is still absent during development. The earlier samples used in these experiments (P3) presented a structure with only the superficial layers clearly defined. The deeper cells in this early cortex (below layer ~II and ~III) show a disorganized distribution, with no clear separation in layers. In the P12 brain it is possible to see some organization of layers, which may be considered almost complete in P14 (Figure 8).
Figure 8: Developmental changes in Evolution in the cortex from P3 to P14 days of development. (A) Position of the section used for all cortical measurements. (B) Sections of the cortex of P3, P4, P5, P12 and P14 brains, ~ 500 microns wide, aligned on the deeper limit of the cortical plate (horizontal white line), 4x magnification. (C) Same layout of (B) but inverted and desaturated to produce a better image. Scale bar 500 µm.

The increment in size of the cortex is coincident with the increase in size of the deeper layers of the cortex. The superficial layers remain constant in size once they are formed and the posterior increment in cells and size is produced in the deeper layer of the cortex, which seems to proliferate before differentiating into a different layer.

Another interesting difference is the integration of the layer VI with the cells of the subplate region with time. While the subplate region is clearly defined in P3, P4, and P5, in P14 that region looks quite similar to the layer VI, and the limit between them is rather diffuse.
Figure 9: (A) P4 gerbil brain coronal section. This section includes the entire cortical plate and the subplate region. The yellow arrow indicates the subplate region. (B) The same sectioning in a P12 gerbil brain. The yellow arrow shows the same subplate region, but it is smaller than P4 and the limits of the same are more diffuse. 4 x magnification. Scale bars 500 µm.
Changes on the size of the cortex.

The measurements of the thickness of the cortex showed a sustained increase in the thickness of the cortex during development. However, those increments have higher magnitudes during the early ages (P3-P5) compared to the older ages (P12, P14) (Figure 10).

Figure 10: Thickness of the cortex over time. The graphic shows the increment on the thickness of the cortex during development. The fitted curved is used for a better representation of the tendency suggested by the data: The growth is more intense in the early stages (P3 to P5) compared with the younger animal (P12, P14)

In order to discard any artifact produced by the tracer, the cortex thickness measurements were registered from both sides of the brain (tracer vs no tracer sides) of the same slice. The results showed some differences between the two cortices, but the increment of thickness remain constant and similar in both sides.
The differences in the measurements from both sides were not significant: Once the curves were fitted, the Wilcoxon Rank test showed no difference between them (Figure 11).

**Figure 11:** Representation of the thickness of the cortex at the side of the tracer (red solid circle) and the opposite side of the brain in the same slice, without tracer (blue triangle). The fitted curves represents the function formed by the measurements points, and there is not difference between them (Wilcoxon Rank test).
Labeling of subplate neurons.

The analysis of the samples from younger animals, P4-P5 ages, showed the labeling of a population of neurons located under the deepest layer of cortex and different to the axonal tracts evident in the white matter connecting the thalamic structures (Figure 12). This tract runs parallel to the surface of the cortical plate it seems to be connected not only to the cortical plate but also to the subcortical level (MGB).

Figure 12: A) Labeling of the subplate cells. The figure shows a P3 brain showing a conspicuous labeling in the subplate region (red arrows). Notice the tracer not only connects with the subcortical connections but also spreads towards the subplate region. (B) Shows the labeling of the same region (red arrows) but ~ 1.5
mm away from the position of the tracer, in a slice more rostral. 4x magnification:
Scale bar: 1000 µm.

This population of cells travels in the horizontal plane and it was detected more than 1.5 mm away from the original position of the tracer, a long distance in rostro-caudal fashion. Based on the results by Kanold et al (2010), this population of cells corresponds to the subplate neurons. The labeling of these cells showed a population of small and heterogeneous cells (Figure 13) and it is present in P3, P4 and P5 brains and even in the P12 brain. The P14 brain showed a direct labeling from the cortex to the MGB.

Figure 13: (A) Labeling in the subplate region. P4 brain showing a clear labeling in the subplate region, just like in P3. (B) The inset shows a group of cell in the
The subplate region is also subject to changes during development. Its thickness varies with its position on the brain in rostral-caudal orientation, and also with its position in the dorsoventral position. The measurements registered in the same region indicated in Figure 14 showed that despite the variability of that region, it seems to experience a period of expansion (P3 to P5) and later a period of possible contraction or integration with the upper layers. (P12 to P14).

Figure 14: Thickness of the subplate region over time. The graphic show the measurements of the subplate regions over time. (A) Region of the cortex measured (red arrow). (B) The thickness of the subplate region increases in early ages but later reduces its size. Scale bar in (A): 500 μm.
Discussion

Considering the differences in size and weight of the brain during development and the adult brain, it was possible to presume a big difference of position and size of A1 in the young gerbil compared to the adult animal. Despite those differences, the proportional positions of the anatomical references used in this study seemed to be consistent with the adult configuration. The results showed that the position of A1 in the developing gerbil has the same positional relationship with the vasculature seen in the adult. Another interesting fact is that the position of A1 based on the vascular references was coincident with the most lateral point of the temporal lobe. This position was evident in the photographs of P3, P4, P5, P12 and P14 brains, taken before the brains were sliced. This new topographic point can be used as another reference to locate the auditory cortex in perfused brains and to complement in vivo experiments (spontaneous activity recordings, thalamico-cortical stimulation, calcium imaging).

The distance from the occipital and frontal lobes to the tracer (putative A1) position increases proportionally in both directions, but it does not mean that the brain growth is uniform. Nevertheless, the distances measured, and also the increments on the cortex thickness, suggest a growth process which is more intense in the early stages of development than in the older ages sampled. These results are consistent with Rübsamen et al results (1), which also showed
the brain growth process is not uniform in space and more intense in the first
days of life.

The Neurovue tracer produced an excellent labeling of the neurons and
axonal tracts in the sampled brains. After 4 weeks the labeling of MGB was
evident in all samples, but the extension of the labeling was not the same in all
the samples. This difference of labeling is likely to be related to the time with the
Neurovue loaded in the sample, and the size of the brain and distance between
the tracer and the MGB, because in all brains sampled, the axonal tracts
revealed by the tracer have the same orientation in all samples.

One interesting fact is the distance covered by the tracer. The surface
labeled in the cortex showed a shorter extension than the distance traveled by
Neurovue to reach the MGB. We could assume that the tracer labeled only the
structures directly related to the A1. The cortical surface labeled may represent
the connections of A1 with the MGB in one way, and the connections of A1 within
the cortex.

The measurements in both sides of the brain samples showed the
Neurovue did not affect the anatomy of the brain in the fixed brain. The use of
these lipophilic dyes represented an important progress in the study of neural
connections; however, there is a limitation: they cannot reveal the direction of the
tracts labeled. In this case the Neurovue did not allow distinguishing whether the
connections were cortico-thalamic or thalamo-cortical. This technique will not provide that information.

The evolution of the layers in A1 and the increase of thickness of the cortical plate seem to be related to the proliferation of the basal strata of the cortex. The superficial layers are formed before the deeper ones, and the underlying cells provide the population for the differentiation of the next layer. This seems consistent with the results by Yu et al (19), showing the synapses in the cortex are produced preferentially between cells in the basal layers of the cortex and cells which migrated from that region towards upper layers.

The labeling of the subplate cells is present in all the samples except P14. This age is considered the moment of the onset of hearing in the gerbil. That may be the reason for the absence of the labeling of that population of cells at that age: the thalamo-cortical auditory pathway can be fully configured at this age, and the connectivity between A1 and MGB may be as adult or mature as possible. This is consistent with the reduction of the size of the subplate region observed in this study. Nevertheless, it is not possible using these methods to determine whether that reduction in size is actually a contraction of that space or, more likely, the integration of the cells in that subplate region to the cortical plate (layer VII or VIb).
CONCLUSIONS

- Anatomical references can be used to determinate the location of auditory cortex during development, before the onset of hearing. The correct position of A1 is given by the center of the blood vessels circle formed by the mid cerebral artery and the inferior cerebral vein in the temporal lobe of the developing gerbil. The point mentioned above is also related to the most lateral point of the temporal lobe of the brain.

- The growth process in the gerbil’s brain does not seem to occur uniformly in the entire organ. Nevertheless, the proportional positions of anatomic references are already configured as in adulthood.

- The thickness of the cortex has a high increase rate before ~P7. This increment becomes slower as the onset of hearing is reached.

- The subplate layer cells are labeled as part of the auditory pathway in the developing brain. This is evident in P3 to P12 brains, but absent in the P14 sample, suggesting the connectivity function of that population of cells is not required after the onset of hearing.
FUTURE DIRECTIONS

Increasing the sensitivity of the model using different Neurovue colors.

One possible improvement on this experimental design for the auditory cortex anatomy and localization is the opportunity of labeling with more precision the entire primary auditory cortex. Using different Neurovue colors (red, green and blue for instance), the entire area of auditory cortex can be traced, including its upper and lower limits. The color differentiation of the tracer will allow to determine which neurons connect to MGB and which ones belong to another cortical area with more precision.

A variation of this approach will be to produce the labeling using different colors of Neurovue starting in different positions: i.e Neurovue blue installed in the MGB and Neurovue Red installed in A1. It may reveal the direction of the tracts labeled, in the case the two (anterograde and retrograde) tracts are labeled separately, and therefore providing one solution to the limitation of this study, or it may produce a mixed labeling. In this case, the point of intersection of the different colors may still provide some interesting information about the involved pathways.
Confirmation of the method using electrophysiological recordings.

Based on the technique described by Ping et al 2007, it could be possible to place an stimulating electrode in the MGB in vivo and record the time-locked response in the A1. This experiment can provide the same information obtained with the tracer, but in vivo. Although the present study has shown that anatomical references are reliable to detect the position of A1, this approach in vivo can provide a higher precision of the limits of A1.

Evaluation of development of the thalamocortical projections.

The same data presented in this study could be used to describe the neuronal connections present in the auditory cortex during development. The images presented in this study show not only a vertical connectivity cortex-thalamus (or vice-versa, not possible to determine in this model) but also an horizontal distribution of neuronal tracts. These connections are very likely to be modified and reconfigured during the ages analyzed in this study.
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