# Electrophysiological studies of the production and cortical representation of vocalisations in the guinea pig

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## Abstract

*Vocal production:* Guinea pigs (GP) are gregarious animals with a well-characterised repertoire of 11 vocalisations. These are context dependent, communicating information about danger, identity and emotional state. Vocalisations have previously been produced by electrical stimulation of three areas in the GP brain: anterior cingulate, hypothalamus and periaqueductal grey. These vocalisations were reported as natural-sounding, but with little or no spectral analysis to support this assertion.

I elicited calls from urethane-anaesthetised GP by stimulating all the above areas, and from the amygdala and several thalamic nuclei. The spectrotemporal properties of these vocalisations were analysed and eight distinct vocal patterns were identified. For comparison, recordings of spontaneous calls from the same colony (Grimsley et al., 2012) were analysed in the same way, then used to name the electrically elicited calls. For six call types the matches between electrically elicited and spontaneous calls were unambiguous. The remaining two elicited calls were identified as being slightly unnatural versions of one spontaneous call.

Five calls were produced during the (1.6 s) electrical stimulation and three were produced after the stimulation, lasting up to 30 s. Concurrent bilateral stimulation of loci producing post-stimulus calls always had an additive effect, whereas stimulation of two loci giving during-stimulus calls was more complex.

*Auditory representation of vocalisations:* To date, eight functional areas of GP auditory cortex have been identified using electrophysiology, and their responses to vocalisations has been investigated previously. I have discovered a new area, ventral to those currently described, which was named deep ventrorostral belt (dVRB). It is

unresponsive to a broad range of puretone auditory stimuli, yet is highly selective to conspecific vocalisations. Single neuron recordings were taken from dVRB and the primary auditory region (AI) during the audio-vocal study.

*Audio-vocal interactions:* The vocal production system communicates the expected sensory consequences of its action. This allows the auditory system to discriminate between self-produced and external sounds. These sensorimotor connections originate in premotor areas of the midbrain as well as motor planning areas of neo- and paleocortex. The basal amygdala (BA) – an emotion-mediating structure – yields vocalisations in GP when stimulated, and is involved in the affective prosody of human speech. It was hypothesised, therefore, that BA would also have an audio-vocal role. A protocol was developed to combine electrical stimulation in BA with auditory presentation of a range of GP vocalisations, whilst recording neural activity in AI and dVRB. In both cortical areas, single-neuron responses demonstrated a complex interaction of electrical and auditory stimuli; showing both enhanced or suppressed responses, depending on call type.

## Declaration

This thesis is the candidate's own work, and has not, whether in the same of different form, been submitted to this or any other university. All microstimulation and physiological experiments, and data analyses were performed by the candidate. The histology was performed, in part, by the candidate. Approximately half of the histology was performed by Zoe Thompson, Histological Technician, Institute of Hearing Research.

# Abbreviations

N.B. a separate abbreviation list is provided in section 3, pertaining to the histological figures contained therein.

| | |
|---|---|
| AC | Auditory cortex |
| ACC | Anterior cingulate cortex |
| AF | Auditory fovea |
| AI | Primary auditory cortex |
| Area S | Small area of AC |
| Area T | Transition area of AC |
| AV | Audio-vocal |
| BA | Basal amygdala |
| CF | Characteristic frequency |
| CN | Cochlear nucleus |
| DC | Dorsocaudal area |
| dlPC | Dorsolateral prefrontal cortex |
| DSC | Dopler shift compensation |
| EC | Efference copy |
| ECoG | Electrocorticography |
| EEG | Electroencephalography |
| $F_0$ | Fundamental frequency |
| fMRI | Functional magnetic resonance imaging |
| GP | Guinea pig |
| HI | Hearing impaired |
| IC | Inferior colliculus |
| IEG | Immediate early gene |

| | |
|---|---|
| LP | Lateral parabelt of primate AC |
| MEAR | Middle ear acoustic reflex |
| MGB | Medial geniculate body |
| NA | Nucleus ambiguus |
| NH | Normal hearing |
| NMV | Motor trigeminal nucleus |
| NVII | Facial nucleus |
| OCS | Olivocochlear system |
| OHC | Outer hair cell |
| PAG | Periaqueductal grey |
| RF | Lateral pontine reticular formation |
| RMI | Response modulation index |
| RMS | Root mean squared |
| SM | Selective mutism |
| SMA | Supplementary motor area |
| STG | Superior temporal gyrus |
| TC | Toothchatter |
| USV | Ultrasonic vocalisation |
| VH | Ventral horn of the spinal cord |
| VPL | Vocal production learning |
| VRB | Ventrorostral belt |
| dVRB | Deep ventrorostral belt |
| VUL | Vocal usage learning |

# Contents

# Introduction

This thesis explores the neurobiology of mammalian vocal production and the effects of this system on the representation of sounds by the auditory system.

Vocalisations are the product of precise, coordinated muscular activity, under the control of motor and pre-motor areas of the brain. This is the final stage in a hierarchy of neural activity. *Whether* a vocalisation is produced, and *what type*, is controlled and influenced by a number of brain regions. This network, ranging from brainstem to neocortex, will be discussed.

The connections between the vocal production and the auditory systems are reciprocal, numerous and occur at every level of the brain. Here, review will focus on the pathways by which the vocalisation system modifies the neural representation of auditory input. These interactions allow the discrimination between self-produced and external sounds.

### 1.1 Vocal production in humans

Human infants usually utter their first word within one year of birth. They continue to learn new words until, around one year later, they are able to demonstrate an understanding of grammar by combining two words in a meaningful way. By the age of 6 years a vocabulary of over 2000 words has been acquired (Ervin and Miller, 1963). Babies, however, produce nonverbal vocalisations from birth. Indeed, a healthy baby will announce its arrival with a prolonged period of crying. Babies have an innate repertoire of 12 vocalisations (figure 1-1) which are present from birth, though for the first 2 months, only the 'cry' is capable of conveying emotions accurately (Scheiner et al., 2006). Wasz-Hockert et al. (1968) subdivided the cry call into: birth, hunger, pain and pleasure. Each has a distinct spectral content and, with the exception of the birth cry, reliably signals its associated emotion. 'Hunger' and 'pain' are present from birth, whereas 'pleasure' does not appear until 3 months of age (figure 1-2). Naïve caregivers are able to distinguish between these cries at above chance levels and improve with training (Keller and Schölmerich, 1987).

During the initial two months the other vocalisations are produced seemingly at random and not as a result of any environmental stimulus. The same is true of apparently emotional facial expressions. This is probably due to the lack of emotional development. There is some disagreement as to the exact rate and progression of emotional development, mainly due to imperfect testing criteria and babies lack of ability to self-report (Giblin, 1981; Izard and Malatesta, 1987; Malatesta-Magai, 1991). It is generally agreed, though, that newborns have two emotional states: aversive and non-aversive. Within three months these emotions develop and refine to give: distress, interest, joy, sadness, disgust and anger. And at around six months fear and surprise appear (Scheiner and Fischer, 2011).

Studies comparing normal hearing (NH) infants with hearing impaired (HI) infants have been useful to elucidate the effect of auditory input; from both the environment, namely mother's voice, as well as feedback of their own voice. In the HI group the hearing impairment was profound, with >100dB hearing loss in both ears (Scheiner et al., 2006). With the exception of 'babble', there were no differences between the two groups in the spectral properties of the calls or the age at which they appeared. Both groups varied their calls in the same ways to express different emotions.

There is some evidence that 'cry' calls have a learned component. A study of 30 French and 30 German newborns showed a small, yet significant difference in the inflection of their cries, with the German babies putting more energy into the initial portion of the cry. This is reflective of the language they heard *in utero* (Mampe et al., 2009). The spectral properties of crying have been shown to vary over the course of a single bout of both the hunger cry (90s) (Zeskind et al., 1993) and the pain cry (4min) (Wood, 2009). In contrast to the linguistically reflective cries, these changes are likely to reflect changes in intensity of affective state.

Whilst there may be a learned component to vocal production, the normal development of the full repertoire is independent of auditory input. This means that their production is innate and genetically pre-programmed.

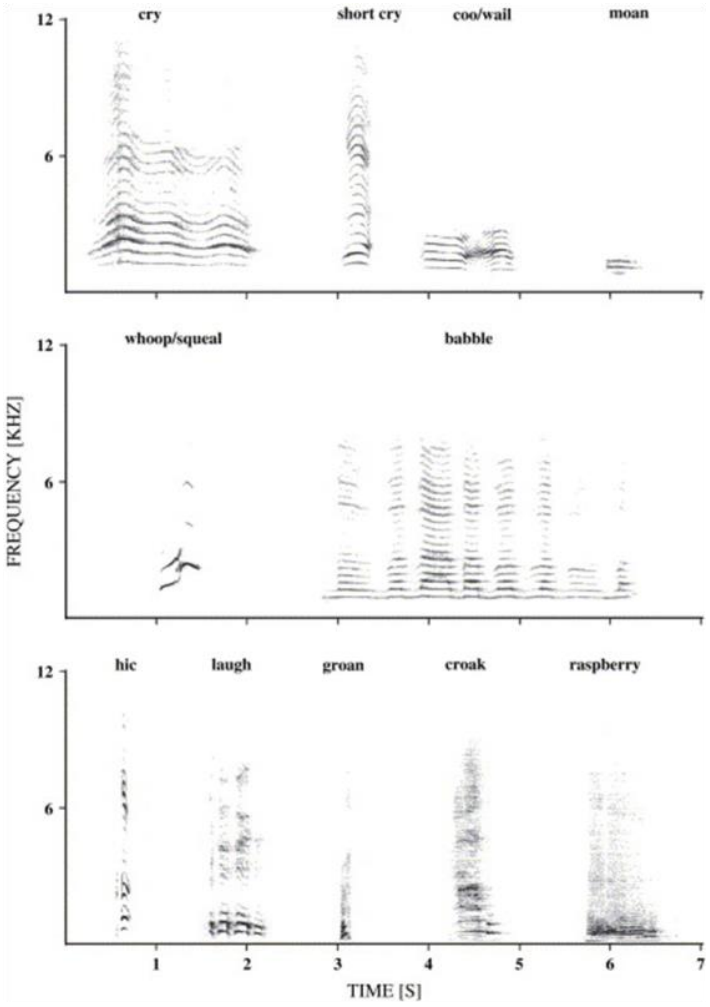



**Figure 1-1** The repertoire of infant calls. All are given during both positive and negative emotional states (Scheiner et al., 2006).

In HI infants 'babble' appears either later or fails to occur (Scheiner et al., 2006). As distinct from the other nonverbal vocalisations, babble is strongly dependent on auditory feedback. It is widely believed that 'babble' is the precursor of phonemes – the phonetic units that make up all the words in a given language (Eilers and Oller, 1994; Oller et al., 1994). In the English language there are 38 such phonemes (Ervin and Miller, 1963). Auditory feedback helps the child to experiment with producing the sounds and auditory input from the mother's voice provides example sounds which they can attempt to copy (Scheiner et al., 2004).

Of the 12 vocalisations present in infants, all except babble develop into the range of nonverbal vocalisations present in adults (Scheiner et al., 2002). As cognitive abilities develop, sensitivity to social group dynamics allows the expression of a wider range of emotional states (Hammerschmidt and Jürgens, 2007). After infancy, we retain the capacity to produce nonverbal vocalisations unconsciously, yet we can also consciously suppress them. Actors regularly produce such vocalisations at will, though this does not necessarily involve the same neural processes.

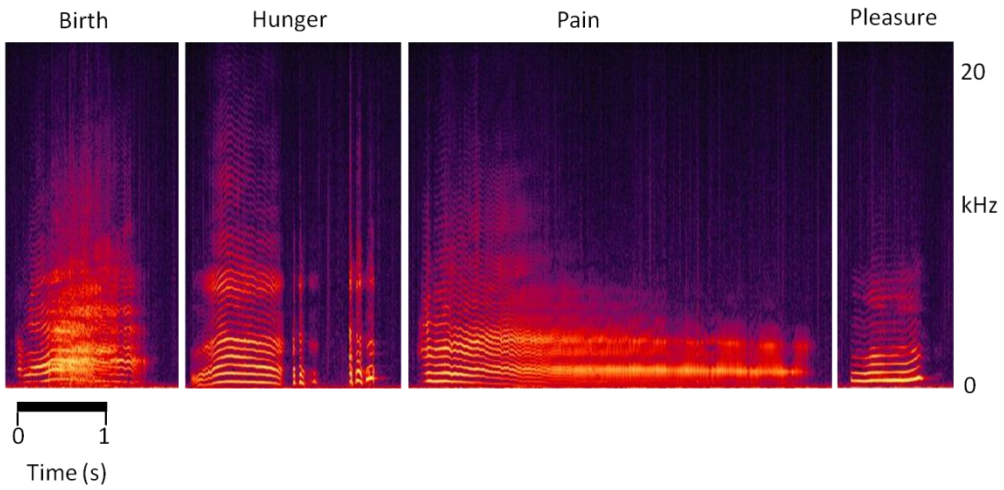



**Figure 1-2** Modulations of the infant cry according to emotional state (Wasz-Hockert, 1968).

### 1.2 Emotional prosody

Emotional prosody is the expression of emotion through the intonation of spoken language. This section will present evidence that the brain network responsible for producing the innate nonverbal vocalisations described above also produces the nonlexical components of affective speech. N.B. emotional/affective prosody is

distinct from linguistic prosody - an aspect of semantic language, such as the rising intonation at the end of a question.

Clinical studies of patients with brain lesions have, historically, provided the first evidence to link brain areas to function, such as the link between Broca's area and language production (Heiser et al., 2003). From clinical studies we know that it is possible for a patient to have a defect in, or even a complete absence of, the ability to produce semantic spoken language whilst maintaining the ability to produce nonverbal vocalisations (Caramazza and Berndt, 1978). In other cases, it is possible for both of these functions to be abolished by a single lesion (Gruber-Dujardin, 2010). These suggest that 1) there is a network for the production of nonverbal vocalisations which can function independently of the network for spoken semantic language and 2) there are aspects of these networks which are shared.

Conversely, it is possible for discrete brain damage to allow the production, as well as the comprehension of semantic language, whilst removing the ability to produce nonverbal vocalisations. One stroke patient (Jürgens and von Cramon, 1982) with a bilateral lesion in the anterior cingulate was able to respond to questions and repeat statements, but could not produce nonverbal vocalisations voluntarily, although he would respond with a cry in the event of a nociceptive or startling stimulus. He had a disinclination to speak and would never initiate a conversation. Furthermore, his speech was rendered monotonous even when asked to repeat statements using emotional speech. This evidence supports the concept of independent networks for semantic language and nonverbal vocalisations. Moreover, the lack of appreciable intellectual disabilities in this case strongly suggests that the circuits responsible for production of emotional prosody are the same as those for nonverbal vocalisations.

Expressive and perceptive prosodic defects were apparent in patients with right hemispheric lesions, whose language abilities were unaffected. This led Ross et al. (1981) to propose a model in which prosody, at the level of cortex, is lateralised to the right hemisphere. As supported by the clinical cases, the right hemisphere equivalents of Broca's and Wernicke's areas were suggested to control, respectively, production and perception of prosody. More recently, however, prosody production defects have been described in patients with brain damage to the left hemisphere only (Schirmer et al., 2001). These have subtly different symptoms to the right hemisphere aprosodias, indicating that in normal function prosody is a bihemispheric product.

In contrast with investigations into perceptual prosody, only one study has used fMRI to study production of prosody in normal adults (Dogil et al., 2002). Though the inherent movement involved in producing vocalisations leads to methodological problems, these can be sufficiently (though not completely) overcome using a sparse imaging protocol. The primary concern is how to get subjects to produce prosody in an authentic, unconscious way. This study fell somewhat short of that ideal. They produced a baseline by asking subjects to speak a string of the same syllable in a monotone, they were then asked to repeat audio samples in which one of the tones was altered to be happy or sad. The study found only cortical activation in relation to these tasks, with bilateral activation of the superior temporal gyrus (STG). The lack of activation in the limbic system and midbrain led them to conclude that the production of prosody is solely under the control of cortical areas. This, however, is in direct contradiction to the lesion case study which implicated anterior cingulate, a component of the limbic system (Jürgens and von Cramon, 1982). Further evidence against the validity of this study comes from clinical stroke cases. Some patients with cortical lesions are unable to smile on demand, yet are still able to produce a natural

smile following an emotional encounter (Kappos 2010). A similar distinction in brain function between 'naturally' produced and 'acted' vocalisations probably exists.

The problem of how to faithfully reproduce the unconscious nature of emotional prosody under test conditions is shared by those using fMRI to study prosody's perceptual system, albeit to a lesser extent. Audio samples presented to participants can be selected to convey emotions in an appropriate way (Adolphs et al., 2002), but the listener cannot be said to be receptive to them in a natural way. For example, in some studies subjects are asked to attend to either the prosodic or linguistic aspects of the sample (Buchanan et al., 2000). Also, if the audio sample is saying something in a, say, angry manor, a normal subject will recognise the intended emotion whilst, at the same time, being aware that the voice is not really angry at them.

### 1.3 Vocalisations in nonhuman mammals

Studies in humans are limited by both the small number of clinical cases presenting with aprosodias and the inherent disadvantages of fMRI studies. For these reasons, it is useful to study an appropriate animal model. All mammals use vocal communication calls to convey information to members of their species (conspecifics). These are known, therefore, as conspecific vocalisations.

There is a wealth of data supporting the assertion that the same networks involved in the production of conspecific vocalisations in nonhuman mammals are the same as those which control nonverbal vocalisations in humans. These can be conceptually divided into five sections, in ascending hierarchical order:

1) The coordinated muscular activity driven by motor and premotoneurons.
2) The gating function provided by the midbrain periaqueductal grey (PAG).
3) Input from the structures regulating emotion and motivational state.
4) The volitional initialisation of vocalisation by the anterior cingulate cortex (ACC).
5) The production of learned vocal patterns, requiring the involvement of neocortex.

The nuclei associated with these stages are shown in figure 1-3.

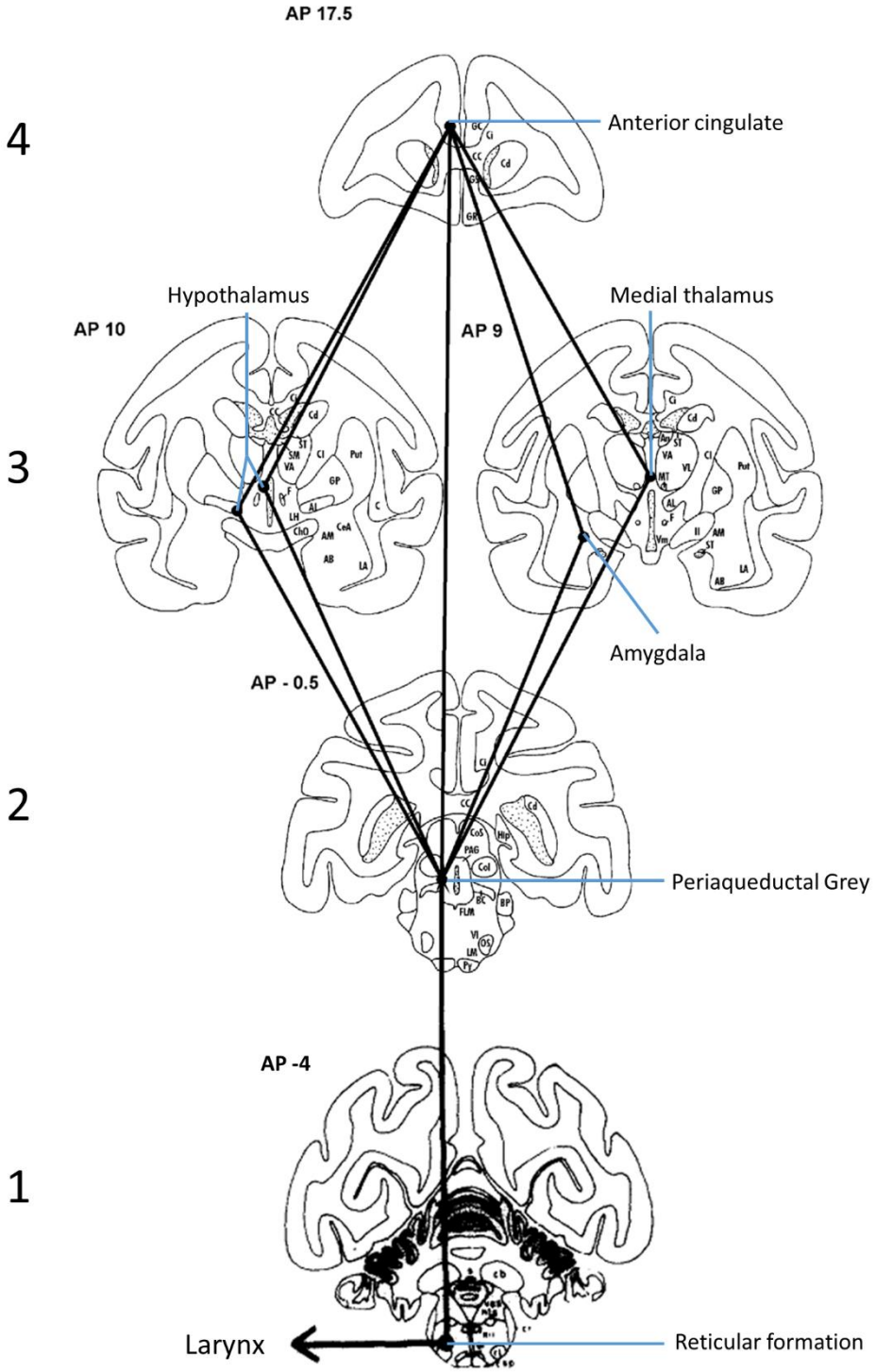



**Figure 1-3** Coronal sections of squirrel monkey brain showing the nuclei associated with the hierarchical order described in the main text. Black dots indicate loci from where vocalisations can be elicited with electrical stimulation. Black lines represent direct descending projections as determined by anatomical tracing. Numerals indicate the position on the hierarchy of vocal production described above. Adapted from Jürgens and Ploog (1981) and Jürgens (1998).

### 1.4 Motor and premotor neurons

For a vocalisation to occur, three muscle groups - respiratory, laryngeal and articulatory - need to be activated in a precise manner. Figure 1-4 shows the locations of major motoneuron nuclei in the pontine brainstem and the muscles that each nucleus innervates.

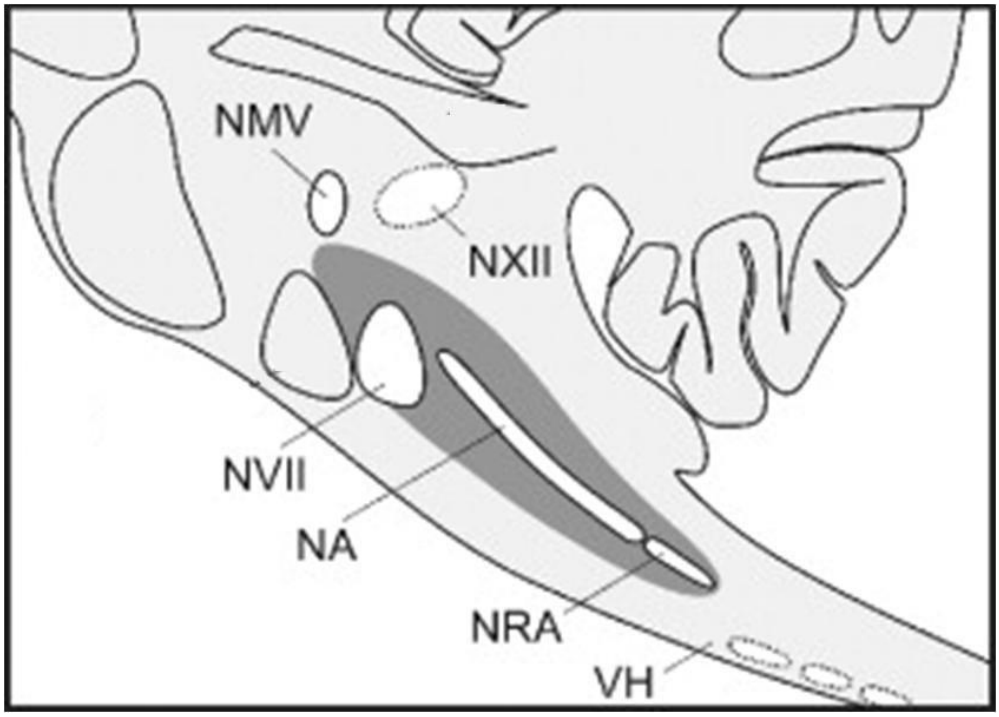



**Figure 1-4** Phonatory motor and premotoneurons.
Motoneuron pools with their respective muscle groups in parentheses: NMV Motor trigeminal nucleus (Jaw); NVII Facial nucleus (Lips); NXII Hypoglossal nucleus (Tongue); NA Nucleus ambiguus (Larynx); VH Ventral horn of the spinal cord (Respiratory).
Premotor neurons of the lateral pontine reticular formation (RF) (dark grey area) act to coordinate the activity of the motoneurons. Adapted from Hage (2010).

The motoneuron pools which drive each muscle group are unconnected (Hage, 2010; Kappos and Mehling, 2010; Shiba, 2010). The coordination must, therefore, reside

upstream in the neural circuitry. Several premotor regions have been identified that connect with all the phonatory motoneurons and may serve as pattern generators for motoneuron activity. A strong candidate is the lateral pontine reticular formation (RF) (Jürgens, 2002). Electrical stimulation of RF most often elicits muscular activation of some of the phonatory muscles without producing a vocalisation. This is because the stimulation cannot sufficiently mimic the necessary precise premotor activity. Stimulation can sometimes yield vocalisations but, for the same reason, they are unnatural sounding (Hage and Jürgens, 2006a). Single neuron recordings in spontaneously vocalising squirrel monkeys highlight areas in RF where neural activity correlates strongly with the spectrotemporal structure of frequency modulated vocalisations. The same neurons are not active during other activities that involve phonatory muscle groups, such as chewing and swallowing (Kirzinger and Jürgens, 1991).

RF receives and integrates sensory information necessary for vocal production. For example, pulmonary stretch receptors indicate the volume of air in the lungs. Bilateral severing of the vagus nerves, which carry this information, prevents vocal production (Ludlow et al., 2008).

### 1.5 The Periaqueductal Grey

In a review paper, Newman (2007) presented a convincing case that all mammalian cries/isolation calls (including those of humans) are controlled by the same mechanisms and serve a similar evolutionary purpose, even though they are sometimes given a variety of names. Newman summarised studies showing that animal vocalizations are generated by neural circuits in the brainstem. The evidence that animals and humans can produce a cry without a cerebral cortex or thalamus

was presented as evidence that no other brain areas are involved. However, the evidence presented in later sections suggests that this model, which implicates only the brainstem, is incomplete.

The midbrain periaqueductal grey (PAG) is an essential component in the vocalisation system. Its destruction, in animals and humans, results in complete mutism whilst leaving the control of respiratory and articulatory muscles unaffected (Newman, 2009). In animals, electrical stimulation of the PAG yields the full repertoire of conspecific vocalisations. Stimulation of PAG in humans has only once yielded a vocalisation: 'euphoric laughter', whilst several other similar procedures reported no vocal activity (Jürgens, 2002).

PAG receives direct input from several higher vocalisation regions, with each producing a subset of the full repertoire. These inputs are glutamatergic and the output is under strong control of GABAergic interneurons (Gruber-Dujardin, 2010). It provides a gating system and regulates the intensity of the vocalisation, with the majority of vocalisation correlated neurons showing activity only before and not during a vocalisation. Of the small number that fire during a vocalisation, all were selective for one vocalisation type and none showed activity correlating with the acoustic structure of the vocalisation (Gruber-Dujardin, 2010).

PAG plays a role in pain perception, behaviour and emotion (Brooks and Tracey, 2005; Gruber-Dujardin, 2010; Vargas and Schenberg, 2001). It receives afferent projections from all the vocal production areas discussed in the following sections and also has a large amount of interconnections. Vocal production loci in PAG are discrete with one locus producing only one vocalisation. Loci show consistency within species, but vary across species (Jürgens, 2002).

Subregions of PAG are associated with different behaviours. Stimulation of dorsolateral PAG elicits fight/flight behaviour in rat and rabbit, and injection of glycine receptor antagonists has anxiolytic effects in rat (Matheus et al., 1994). Pharmacological activation of lateral PAG in rat and cat caused attack behaviour, in contrast to the escape behaviour shown by control animals (Gruber-Dujardin, 2010). In humans, electrical stimulation of dorsal PAG evokes fear, whereas stimulation in other areas (not declared) can evoke euphoria (Drevets, 2001). Importantly, in squirrel monkey, it was shown that the types of vocalisation elicited by stimulation did not correlate with the emotional state measured by a behavioural test. Therefore, the PAG vocalisation network is involved in direct initiation of vocalisations. This is supported by the fact that the latency of PAG elicited vocalisations is very short (~50 ms) (Jürgens, 1976).

**1.6 Hypothalamus**

The functions and connections of the hypothalamus are numerous. Its role in the neuroendocrine system contributes to body temperature, hunger, thirst and sleep/wake cycles (Leibowitz, 1971; Myers and Yaksh, 1969; Nitz and Siegel, 1996).

Electrical stimulation in this area has yielded conspecific vocalisations in guinea pig (Martin, 1976), squirrel monkey (Jürgens, 1976), macaque (Robinson, 1967), cat (Kanai and Wang, 1962) and rat (Yajima et al., 1980). Vocalisations representing a range of motivational states can be elicited.

In particular, studies using rat and squirrel monkey found locations in the lateral hypothalamus (rat) and ventral and periventricular hypothalamus (squirrel monkey) where stimulation would elicit a vocalisation associated with a positive emotional

state. When these animals were given the opportunity to self-stimulate, they did so repeatedly (Burgdorf et al., 2007; Jürgens, 1976). These data suggest that the production of the vocalisation is a secondary effect of stimulating a reward centre. That is, the emotional state is produced first, followed by a matching vocalisation. This assertion is supported by the long latency of electrically stimulated vocalisations (~400ms)(Jürgens, 1998).

In humans, electrical stimulation of hypothalamus has been used to treat cluster headache and appetite disorders though there are no reports of vocal production (Perlmutter and Mink, 2006). There are reports of speech deficits following stroke-related damage to hypothalamus (Garg et al., 2000). In these cases, however, the damage was widespread, including several other brain areas. There are no reports of selective hypothalamic lesions affecting speech production. Reports of epileptic seizures characterised by bouts of laughter and feelings of mirth have been shown to have a hypothalamic involvement. However, in these cases surgical intervention to the hypothalamus failed to completely eliminate symptoms, and it was suggested that multiple brain regions are involved (Arroyo et al., 1993).

The hypothalamus receives direct input from vocal production loci in ACC (Jürgens and Ploog, 1981). It may receive input from the amygdala and stria terminalis (see section 1.7). All subnuclei of the hypothalamus send strong projections to the PAG (Dujardin and Jürgens, 2005). Retrograde tracing studies of vocalisation producing PAG loci of squirrel monkey and guinea pig agree that the strength of connections to hypothalamic subnuclei correlates with the aversive/positive vocal types. Posterior hypothalamus has strongest connections with ‘aversive’ vocalisations, whereas lateral hypothalamus is connected most strongly with ‘positive’ vocalisations (Dujardin and Jürgens, 2006; Kyuhou and Gemba, 1998).

**1.7 Amygdala**

Vocalisations have been elicited by stimulation of the central, medial and basal nuclei of the amygdala in cat, pig, macaque, squirrel monkey (Jürgens, 1982; Manteuffel et al., 2007). Since the amygdala is commonly associated with fear and anxiety it is unsurprising that all these studies elicited vocalisations associated with these emotions. Only in the squirrel monkey, however, was it possible to also elicit vocalisations corresponding with confidence and group bonding. Here, the loci were not spatially separate; different vocalisations could be elicited using different electrical stimulation parameters, leading to the assertion that different cell types are preferentially activated by specific parameters.

Researchers have previously disagreed on the route for the descending pathway from amygdala to PAG (De Molina and Hunsperger, 1962; Hilton and Zbrożyna, 1963). A study by (Jürgens, 1982) reconciled these by providing evidence for two separate pathways: 1) the direct amygdalofugal pathway passing via the ventral hypothalamus 2) to the indirect pathway that projects first to the stria terminalis then onwards via the ventral hypothalamus. He was able to abolish just one type of vocalisation with lesions to the stria terminalis, whilst leaving the other intact. A later study (Dujardin and Jürgens, 2005) extensively traced all afferents from vocal production loci in PAG. They showed direct connections with both the amygdala and the stria terminalis. This suggests that both of the amygdala vocalisation pathways pass through hypothalamus without synapsing there. Given both the long latency of vocal production from the amygdala (~400ms) (Jürgens, 1998) and the hypothalamic involvement in emotion mediation it is also possible that both stria terminalis and amygdala also have functional connectivity with the hypothalamus. Reciprocal connections have been demonstrated anatomically (Hines et al., 1985).

As with the hypothalamus, the amygdala is thought to yield vocalisations as a secondary effect. Stimulation of a 'positive' vocalisation is associated with self stimulation behaviour in squirrel monkey (Jürgens, 1976). Furthermore, stimulation in the same electrode location that is subthreshold for vocal production still elicits the behavioural response. The correlation between 'fear' calls and behaviour were not investigated. In human, reports of fear and anxiety can follow amygdala stimulation (Halgren et al., 1978). This agrees with the primate data insofar as a change of emotional state alone can be produced.

**1.8 Midline thalamus**

Stimulation of the midline thalamus has given vocalisations in rat, macaque and squirrel monkey (Jürgens and Richter, 1986; Robinson, 1967; Yajima et al., 1976; Yajima et al., 1980). As with the hypothalamus and amygdala, it is involved with the production of emotion. In squirrel monkey electrical stimulation gives a 'fear' vocalisation which is correlated with matching avoidance behaviour (Jürgens, 1976). Again, suggesting that the vocalisation is a secondary effect following a change in emotional state. In rat and cat the midline thalamus has been implicated in arousal and fear (Montaron and Buser, 1988). It sends numerous projections to ACC (Barbas and Pandya, 1991) and it is possible that this may be a pathway of vocal production (Paus, 2001). A descending pathway is also possible since it projects directly to PAG (Dujardin and Jürgens, 2005).

**1.9 Anterior cingulate cortex (ACC)**

The ACC is involved in the volitional control of vocal production. There are different types of cortex within the cerebrum, so it is important to highlight a distinction: Cingulate cortex is 5-layered paleocortex and represents an evolutionarily older brain structure than 6-layered neocortex. ACC projects directly to the PAG and indirectly via both the amygdala and the hypothalamus (Jürgens and Ploog, 1981).

Electrical stimulation in this region has produced vocalisations in nonhuman primates, cat, bat and guinea pig, but not human (Fried et al., 1991; Gemba et al., 1999; Gooler and O'Neill, 1987; Kaada, 1951; Talairach et al., 1973). Macaque and rhesus monkeys trained to produce a specific vocalisation in order to get a reward were no longer able to do so following bilateral destruction of ACC. These monkeys were still able to perform a lever pressing task at normal levels and would also respond to a painful or startling stimulus with the normal vocalisation (Aitkin et al., 1981; Sutton et al., 1973). This is in agreement with the ACC lesion patient described in section 1.2 who showed a complete lack of willingness to initiate speech. In this case, and with the nonhuman primates, intelligence is unaffected. The same goes for the basic vocal production system, pain sensation and the necessary sensory integration to allow a startle response. Of course, the patient in this case study was still able to speak if instructed. This most likely represents the dominance of neocortical structures in producing learned vocal patterns (i.e. speech). He was unable to produce speech with emotional intonation (Jürgens and von Cramon, 1982), this agrees with the monkeys' inability to volitionally produce a conspecific vocalisation.

Echolocating bats use ultrasonic vocalisations (USV) to navigate their environment and to catch prey. They have an extended portion of the cochlea, known as an

auditory fovea (AF), which gives high frequency sensitivity to the returning echoes. The Doppler Effect causes an increase or decrease in the frequency of echoes depending on whether the relative distance between the bat and its prey is increasing or decreasing, respectively. In order that the USV echoes always arrive at the AF, the bat must adjust its USV frequency. This behaviour is known as Doppler Shift Compensation (DSC) (Kuc, 1994; Rübsamen and Schäfer, 1990). In echolocating bats electrical stimulation of the ACC yields the full range of USVs. The arrangement of USV producing loci is tonotopic, i.e. they are arranged in a spatial/frequency gradient. The ACC is an essential component of the DSC system and provides further evidence that ACC is involved in the volitional aspect of conspecific vocal production (Gooler and O'Neill, 1987; Moss and Sinha, 2003).

**1.10 Neocortex**

Paul Broca was the first to provide evidence that neocortex is essential for the production of learned vocalisations (Heiser et al., 2003). Damage to Broca's area, also known as supplementary motor area (SMA) can, in humans, severely impair speech production (Heiser et al., 2003). Electrical stimulation of SMA elicits vocalisations in humans, but not animals (Fried et al., 1991). In rhesus and squirrel monkey, destruction of SMA has no effect on vocal production (Aitken, 1981; Sutton et al., 1974). In squirrel monkey destruction of both preSMA and SMA has minor effects; only the rate of production of the 'isolation call' was reduced. The spectral structure of this and other vocalisations was unaffected (Kirzinger and Jürgens, 1982). Also, both squirrel and rhesus monkeys with SMA lesions showed no reduction in performance for an operant vocal conditioning.

Squirrel and rhesus monkeys are vocal usage learners (VUL). Both species perform *antiphonal calling* which involves precise syntactic use of innate vocalisations to communicate their identity. This allows groups to coordinate activity when out of visual contact, as is often the case in their natural habitat of the tropical forests of South America (Snowdon, 1989). Single-neuron electrophysiological recordings in awake, freely behaving marmosets have revealed SMA neurons which show activity correlated with either vocal production or vocal perception. A further group of neurons was found to be active only during antiphonal calling (Miller, 2012). Evidence from immediate early gene (IEG) studies corroborates this finding. IEGs are histological markers for neural activity. Increased expression of ERG-1 (Simões et al., 2010) and c-Fos (Miller et al., 2010) was detected in SMA of marmosets following a bout of antiphonal calling.

Vocal production learning (VPL) is another type of vocal learning as distinct from, but not mutually exclusive with, VUL. VPL is the acquisition of vocal components with distinct and repeatable spectral properties. Human speech is, of course, the most sophisticated example but a range of other mammals display simpler VPL behaviour. Direct neocortical to motoneuron projections are essential for the productions of learned utterances (Feenders et al., 2008). It was previously considered that only primates (including humans) have these connections. Indeed, a comparative study of such connections in a range of primates showed more numerous projections to tongue-controlling motoneurons in VPL species than nonlearners. Furthermore, the strength of these projections in VPL species varied approximately in proportion with the sophistication of their VPL abilities (Jürgens and Alipour, 2002). Recently, however, VPL has been observed in mice. Moreover, mice show increased vocalisation-related expression of IEG in motor cortex and direct, although scant, projections to phonatory motoneurons. Advances in histological techniques as well

as automated spectral analysis software are the likely explanations for this late discovery. Destruction of motocortical areas in mice disrupts the production of learned vocal phrases, leaving the repertoire of innate vocalisations intact (Arriaga and Jarvis, 2013).

### 1.11 Guinea pig as an animal model

The guinea pig (GP) (*Cavia porcellus*), a common laboratory animal, is a gregarious and vocal rodent. Compared to other rodents, they have an extensive repertoire of 11 innate vocalisations. Large groups in captivity have been studied behaviourally and been shown to produce specific vocalisations according to environmental factors and emotional states (Arvola, 1974; Berryman, 1976). They are not thought to be vocal learners so are ideal for the study of innate vocal production.

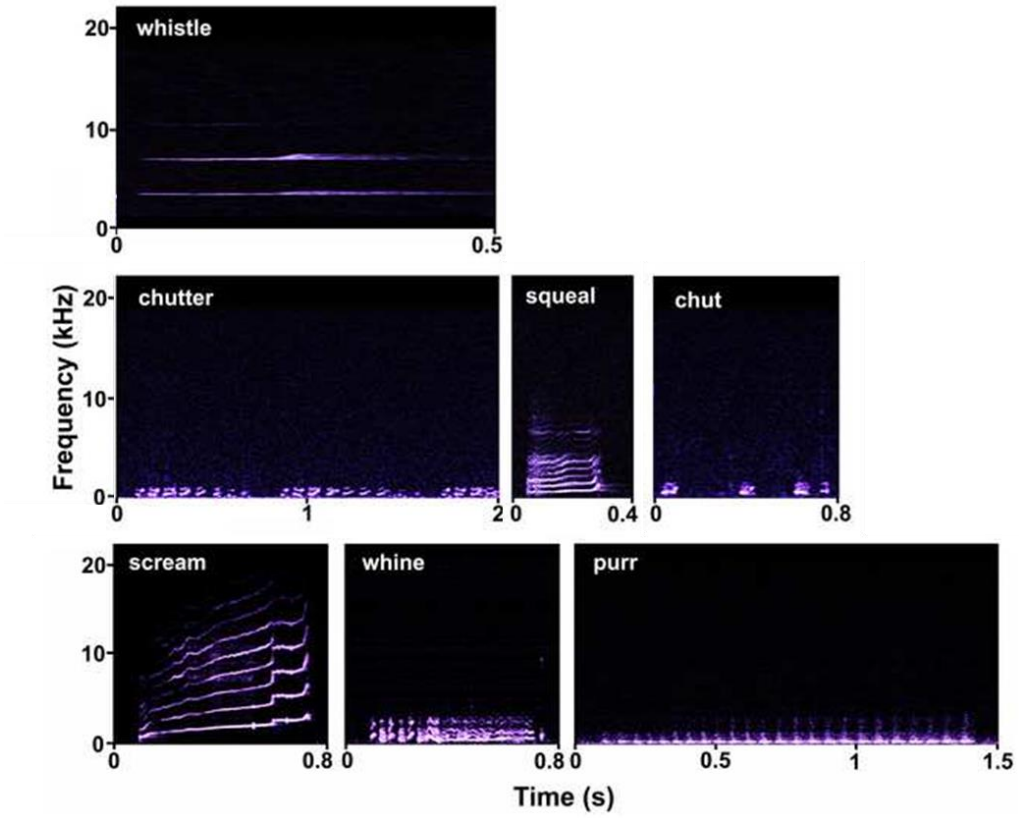



**Figure 1-5** Spectrograms of the seven spontaneous GP vocalisations that are pertinent to the present study (Grimsley et al., 2012).

Figure 1-5 shows spectrograms of the seven GP vocalisations that are important for this study. In addition, there is a non-voiced vocalisation, toothchatter (TC). Rapid jaw movements cause the teeth to strike together, producing a series of broadband clicks (Arvola, 1974). Guinea pig vocalisations are categorised as either aversive or non-aversive and are produced in specific social circumstances, described below (Arvola, 1974; Berryman, 1976).

- Whistle is non-aversive. It is associated with caregiving, being produced by a mother to her young, and by any adult in anticipation of being given food.

- Chutter is mildly aversive. It is produced by females during unwanted advances by male, and is produced by males fleeing mildly aggressive advances of a more dominant male.
- Squeal is moderately aversive. It is produced in response to pain.
- Chut is non-aversive. It is produced during friendly social interactions and whilst exploring a new living space.
- Scream is strongly aversive. It is produced in response to pain or fear. Following an aggressive encounter, the losing animal will retreat to the corner of the cage and scream repeatedly whilst adopting a defensive posture. These screams can become more intense if the dominant male approaches.
- Whine is moderately aversive. It is produced in the same circumstances as chutter. Whine often follows chutter when these interactions occur over a prolonged period of time.
- Purr is non-aversive. It is produced by males and females during courtship and mating. It is also produced by suckling young.
- TC is strongly aversive. It is produced by both genders during aggressive and violent encounters.

A limited number of studies have used electrical microstimulation to elicit vocalisations in GP. Martin (1976) performed a thorough microstimulation mapping in three brain areas of awake, freely-behaving guinea pigs. Several distinct calls were elicited from locations in the hypothalamus and midbrain. Unfortunately, no spectral analysis of the calls was performed, so the names used may be misleading.

Two studies, involving electrical stimulation, by Kyuhou and Gemba (1999; 1998) elicited vocalisations, from the PAG and ACC respectively, in anaesthetised guinea pigs. Anatomical tracing was used to show descending projections from the motor

cortex and ACC to the PAG, as was seen in the squirrel monkey (Jürgens and Ploog, 1981).

There are clear anatomical similarities between the vocalisation circuitry of GPs and primates. This, together with the behavioural studies of social mammals and the similarities in their call structure, suggests that GP is an appropriate model for the study of nonverbal vocal communication in humans.

To date, GP calls have been investigated either as part of behavioural studies of domesticated groups or elicited by electrical microstimulation. Two field studies observed three genera of *Cavia*, the wild relatives of the domestic GP, and noted specific calls with their associated behaviour and emotional states (Rood, 1972; Rowlands et al., 1974). The common theme, however, is that little or no spectral analysis was performed. Most investigators relied on their hearing ability to recognise the differences between calls and the nomenclature is, at best, inconsistent.

For these social animals it is clearly very important to be able to perceive these vocalisations and discriminate between them. Physiological studies have investigated the sensory representations of conspecific vocalisations in the auditory system. This is discussed in section 1.22.

**1.12 Aims of the current microstimulation study**

Initial experiments aimed to elicit vocalisations from the hypothalamus, ACC and PAG, using a lightly anaesthetised guinea pig preparation modified from Kyuhou and Gemba (1998). This was then extended to include the midline thalamus and the amygdala. The ambiguity of nomenclature in the studies described above was

addressed using modern spectral analysis software to quantify the spectrotemporal characteristics and so classify vocalisations in an unbiased manner.

Recordings of spontaneously vocalising, freely moving guinea pigs, from the same colony were analysed in the same fashion. This allowed me to answer two questions: Are particular brain areas associated with specific vocalisations? Do the experimentally elicited vocalisations have the same spectrotemporal structure as spontaneously elicited ones?

A further aim was to assess the potential interactions between vocal production brain regions. A series of experiments was performed using simultaneous stimulation of two different structures, both across and within hemisphere.

**1.13 Auditory-vocal (AV) interactions**

Humans with acquired hearing loss show a gradual degradation in speech performance (Lane and Webster, 1991; Perkell et al., 2007). It is possible for the congenitally deaf to learn to produce intelligible speech but, even in the best cases, it is noticeably impaired (Park et al., 1994). These facts highlight the importance of hearing (auditory feedback) to both acquisition and maintenance of speech production abilities. Speech is the most advanced form of vocal production learning (VPL), as discussed in section 1.11.

Cognitive neuroscientists have used a variety of methods to study normal hearing individuals as well as those with specific speech disorders. Though some valuable insight has been gleaned, the techniques used to study human brains offer far lower spatial and/or temporal resolution compared to those available for animal research. Moreover, the focus of this research places emphasis on the potential cortico-cortical interactions. This section will review the evidence for numerous audio-vocal interactions at all levels of the brain.

Current data support a 'forward model' whereby the vocal production system sends a projection to the auditory system encoding the inverse of the 'expected' auditory input. This is known as an 'efference copy' (EC). If the EC matches the actual auditory input then self-produced vocalisations will be suppressed (Crapse and Sommer, 2008). It is presumed that efferent copies originate in motor and premotor areas of neocortex, although no evidence for this has yet come to light (Flinker et al., 2010). As will be described below, several locations of audio-vocal interaction have been described; spanning all levels of the brain.

Primates and bats have been used to study audio-vocal (AV) interactions at the level of single neurons. As described in section 1.11, marmosets and squirrel monkeys are

not VPL, yet their vocalisation behaviour involves neocortical function, albeit to a lesser extent than in humans (Hammerschmidt et al., 2001; Jürgens, 2009; Tschida and Mooney, 2012).

Echolocating bats are reliant on AV interactions to perform Doppler Shift Compensation (DSC) (Boughman, 1998). This specialised behaviour is not VPL insofar as the modifications to vocal output are continuously being modified in response to environmental changes during flight. Some bat species, however, have been shown to display true VPL, in addition to their echolocation abilities. In particular, the males of several species have been shown to use individual courtship songs (Behr and von Helversen, 2004; Bohn et al., 2009). As yet nothing is known about the anatomy and physiology behind the production of these learned vocal patterns.

The ability to discriminate between self-produced and external sounds is a necessary prerequisite for VPL. Since cetaceans, bats and mice demonstrate VPL, these animals must also have self/external discrimination abilities.

Self/external discrimination, therefore, exists across a disparate range of species; in non-VPL directly and in VPL by implication. Coupled with the fact that anatomical and physiological similarities have been demonstrated between bats and primates, this suggests that the basic self/external discrimination ability exists across the majority of mammalian species.

### 1.14 Evidence from the mammalian central nervous system

Presented below is an overview of the current understanding of AV interactions in the central nervous system. Frequency specific components begin with the

olivocochlear system (OCS). The review will then progress through the ascending auditory system from brainstem to neocortex.

There are two factors, other than those involving EC, that affect the way self-produced vocalisations are perceived. These are the middle ear acoustic reflex (MEAR) and cranial bone conduction. There is also an involvement of the somatomotor system, at the level of brainstem and at neocortex. These will be discussed in due course.

As an animal grows its vocal apparatus also grows, giving changes in vocal output. The AV system will need to account for the fact that different auditory signals are resulting from the same motoneuron activity (Abt et al., 1929). Cheung et al. (2005) surgically altered the larynx of marmosets to permanently lower the frequency of vocal production. Plastic changes occurred in primary auditory cortex over the course of 5 – 15 months to encode the new, lower frequency, self-produced vocalisations. These changes in response properties did not alter the normal tonotopy.

**1.15 Olivocochlear system (OCS)**

The activity of outer hair cells (OHC) is modulated by efferent projections from the periolivary region (POR) and the superior olivary complex (SOC). During vocalisation, the OHCs act to dampen the response of the basilar membrane (Goldberg and Henson, 1998; Liberman and Guinan Jr, 1998). This activity is frequency specific so, in addition to providing protection for the inner ear, the OCS is the first stage at which self-produced and external sounds begin to be discriminated (Hage et al., 2007).

Children with Selective Mutism (SM) are able to speak normally in quiet, but struggle or are unable to speak in noisy environments. The vocalisation induced OHC activity

(as measured by otoacoustic emissions) is significantly less in SM children compared to normal controls, suggesting reduced OCS activity and so reduced ability to perform self/external discrimination (Arie et al., 2007).

**1.16 Subcortical audio-vocal interactions**

Using auditory brain stem responses, vocalisation correlated suppression of the upper brainstem has been shown in human and bat (Papanicolaou et al., 1986). It is clear, however, that at the level of single neurons a more complex set of interactions gives rise to this phenomenon.

Auditory-vocal interactions have been found in spontaneously vocalising bats and primates in the medial geniculate body (MGB) (Olsen and Suga, 1991), the external nucleus of the inferior colliculus (ICx) (Pieper and Jürgens, 2003; Schuller, 1979; Tammer et al., 2004), the periolivary region of the superior olivary complex (Hage et al., 2006) and the ventral nucleus of the lateral lemniscus (Metzner, 1993, 1989). All these areas contain neurons that respond to externally produced vocalisations, but show a reduced or absent response to self-produced vocalisations, thus accounting for the auditory brainstem responses described above. Each of these areas also contains neurons that show *enhanced* response to self-produced vocalisations. Other multi-modal effects have been observed: Some neurons gave an identical response to external and self-produced stimuli and could only be distinguished from auditory cells by the fact that activity was observed in advance of vocal production. Some showed variation in firing pattern rather than rate (Hage et al., 2006). These findings suggest that suppression of self-produced sounds is not the only purpose of these interactions. Furthermore, in POR, three neurons were observed to give selective

modification to just one self-produced vocalisation. Though only a small number, this result is suggestive of a self/external discrimination function (Hage et al., 2006).

All single-neuron studies used small numbers of animals and, in each, audio-vocal neurons made up only a small subset of auditory sensitive cells. This low sample number is compounded by the variety of multimodal responses that have been observed. With current data it is impossible to create a comprehensive model of subcortical audio-vocal interactions.

In cat and GP somatosensory-auditory integration has been shown at the level of the cochlear nucleus (CN) and ICx. Neurons in both the ventral and dorsal CN show either enhanced or suppressed responses to noise during concurrent electrical stimulation of the spinal trigeminal nucleus (Shore and Zhou, 2006). Bimodal neurons in ICx were shown to represent auditory as well as tactile stimuli (Aitkin et al., 1981). These interactions are supported by anatomical evidence and likely subserve the discrimination of *all* self-produced sounds such as chewing, swallowing and breathing as well as vocalisations (Zhou and Shore, 2006). These findings agree with the audio-vocal work described above but could also be the source of confounds.

**1.17 Audio-vocal interactions at auditory cortex**

Electroencephalography (EEG) studies have shown that humans give a suppressed auditory cortical response to self-produced speech. When auditory feedback was pitch-shifted this suppression was reduced. The amount of suppression was inversely proportional to the frequency shift of the feedback (Behroozmand et al., 2009). This fits with the forward model in that a greater difference between expected versus actual auditory input results in a stronger auditory response.

The disadvantage of EEG is that it has poor spatial resolution. fMRI studies, using vocalisation playback as a control, have shown suppressed response to self-produced speech in primary and belt areas of auditory cortex (Allen et al., 2005; Christoffels et al., 2007; Fu et al., 2006). Such studies have the disadvantages of poor temporal resolution and not allowing the use of real-time pitch-shifting.

During neurosurgery it is possible to place electrode arrays directly on the surface of the brain, giving much improved spatial resolution whilst retaining the benefits of EEG. This technique is known as electrocorticography (ECoG). Three auditory studies placed flat arrays lateral to the Sylvian fissure (corresponding with lateral belt areas) (Cho-Hisamoto et al., 2012; Flinker et al., 2010; Greenlee et al., 2011). Each group reported areas showing a range of weakly suppressed and weakly enhanced activity in response to self-produced vocalisations. In two cases this vocalisation was speech. Cho-Hisamoto et al. (2012) recorded from a 10 month old girl and showed that responses to self-produced 'babble' matched what was seen in adults. This finding is in agreement with the assertion in section 1.1 that 'babble' is a precursor of phonemes and is dependent on AV interactions.

Eliades and Wang (2003, 2005) recorded single-neuron activity in primary auditory cortex of freely vocalising marmosets. The majority (~75%) of neurons showed a suppression of firing rate during self-produced vocalisations. This suppression could be removed using pitch-shifted feedback. Figure 1-6 shows a population histogram of all suppressed neurons. The green bar represents the time during which the firing rate was significantly suppressed. Importantly, the suppression occurs in advance of vocal production so this supports the forward model. Some neurons showed enhanced response to self-produced vocalisation. In contrast to the suppressed neurons, enhanced neurons showed increased activity starting at the same time as

vocal onset. Furthermore, the rate modulation for suppressed neurons was greater than for enhanced neurons, leading to the conclusion that the suppressed population had a greater involvement in self/external discrimination (Eliades and Wang, 2005; Eliades and Wang, 2003).

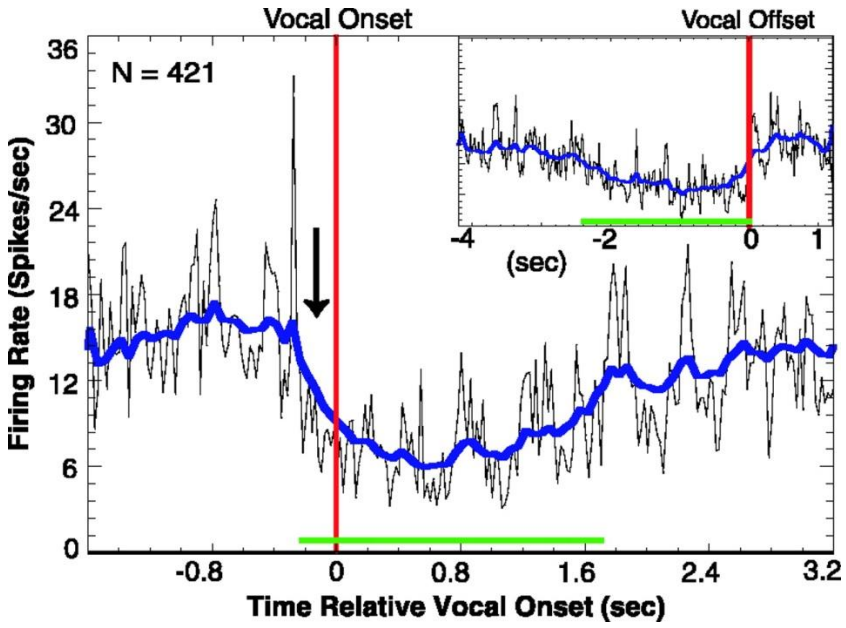



**Figure 1-6** A population histogram of all suppressed auditory cortical neurons. Red lines show vocal onset and offset. Green bars show the time during which the neural response was continuously suppressed. Suppression precedes vocal onset (arrow) yet returns to baseline at the same time as vocal offset. (Eliades and Wang, 2003)

Single-neuron recordings in squirrel monkey are consistent with the marmoset study described above (Ploog, 1981). Also in this species, two studies have investigated the potential origins of these AC modified responses. Using the same technique, Alexander et al. (1976) and Müller-Preuss et al. (1980), suggested roles for, respectively, dorsolateral prefrontal cortex (dlPC) and ACC in EC production. They recorded neurons in the STG whilst electrically stimulating in dlPC and ACC. STG is the

nonhuman primate homologue of the lateral belt region mentioned in the human ECoG study above, and it contains neurons selective for conspecific vocalisations. ACC and dlPC project strongly to STG, and STG neurons were found that respond to electrical stimulation by either increases or decreases in firing rate. Several of these, and surrounding neurons, also responded to auditory stimuli, including conspecific vocalisations.

### 1.18 The middle ear acoustic reflex (MEAR)

The middle ear acoustic reflex (MEAR) is an involuntary contraction of the middle ear muscles, triggered by loud sounds, to protect the delicate inner ear from acoustic damage (Møller, 1974). Vocal production also activates the MEAR for the same reason. In human, cat and bat the MAER is activated just *prior* to vocal onset, suggesting an anticipatory function. The attenuation caused by the MEAR is uneven, with higher frequencies being more greatly attenuated. This modification applies to all incoming sound, not just one's own vocalisation. Therefore it is unlikely to aid in the discrimination between self/external (Carmel and Starr, 1963; Henson Jr, 1965; Salomon and Starr, 1963; Suga and Jen, 1975).

### 1.19 Bone conduction

The sound of one's own voice is received, in part, through the outer ear but also via conduction through the skull (Stenfelt and Goode, 2005). Transmission through bone attenuates lower frequencies less than higher frequencies. It is important to note that, whilst the attenuation of sound is uneven, the frequency components are unaltered and the overall intensity of the auditory input is unchanged (Békésy, 1949).

In contrast with MEAR, this will only affect the self-produced vocalisation. The reason that one's own voice sounds lower during speaking when compared to listening to a recording is a combination of bone conduction and the MEAR (Irvine and Wester, 1974). The effect of bone conduction is negligible when the full range of audio-vocal interactions is taken into account (Eliades and Wang, 2003).

**1.20 Somatomotor system**

The first assertion in this review stated that auditory information is essential for acquisition and maintenance of normal speech abilities (Perkell et al., 2007). Whilst this is true, it is also clear that speech as a learned motor behaviour involves the somatomotor system, which can partially compensate for the loss of audition. Cochlear implant users with their implants switched off were asked to speak whilst having mechanical pressure applied to their jaw. In the absence of auditory input these participants were able to push against the machine in order to maintain correct speech production (Nasir and Ostry, 2008). In human, damage to primary somatosensory cortex can cause articulation impairment (Luria, 1965) and fMRI and PET studies show speech related activity in these regions (Herholz et al., 1994; Lotze et al., 2000; Petersen et al., 1988). In macaque, vocalisation-correlated physiological activity in this region can precede vocalisation by as much as 700 ms (Gemba et al., 1999), suggesting it is part of the 'forward model' described in section 1.13.

**1.21 The importance of audio-vocal research**

The forward model of EC sensorimotor interactions was proposed over 60 years ago. Numerous human behavioural and psychophysical studies across in all sensory

modalities have supported this hypothesis (Griisser, 1995). Little is known, however, of the neural mechanisms that give rise to these behaviours.

As stated, a functioning AV system is essential for language acquisition and maintenance. There are also a number of conditions that highlight AV deficits, such as stammer, autism, schizophrenia and Parkinson's disease (Heinks-Maldonado et al., 2007; Howell et al., 2000; Kiran and Larson, 2001; Russo et al., 2008; Whitford et al., 2012). A more detailed understanding of the AV system is required before treatments for these conditions can be developed.

### 1.22 Sensory representation of conspecific vocalisations

Whether one is passively listening to a vocalisation or actively vocalising, the auditory information that reaches the cochlear is similar. It is useful, therefore, to understand how conspecific vocalisations are represented in the auditory system before considering how this representation is affected by EC during vocalisation. In particular, a review of the GP AC literature is pertinent to the present study.

The GP AC has been characterised, according to its electrophysiological properties, as having eight areas (Wallace et al., 2000). Figure 1-7 shows these areas and their location with respect to cranial landmarks. The majority of ascending auditory inputs reach primary AC (AI) and the dorsocaudal area (DC), both of which are tonotopically organised. From there, auditory information passes laterally to the six belt areas, two of which are tonotopically arranged: the small area (S) and the ventrorosteral belt (VRB). The belt areas tend to have higher latencies, suggesting that they are further along the hierarchy of processing (Wallace et al., 2000).

The majority of the auditory electrophysiological literature has focussed on responses to artificial stimuli such as pure sine waves and broadband noise. Far fewer researchers have used natural sound such as conspecific vocalisations. Grimsley et al. (2012) is the most comprehensive of these, investigating all eight areas in the same study. The authors presented a series of ten conspecific vocalisations to anaesthetised guinea pig whilst recording single-neuron activity. Information theory was used to assess each neurons ability to discriminate between call types. Populations in VRB, AI and S showed the greatest ability to discriminate, in that order. These areas differed, though, in the way that this information was encoded. For example, neurons in VRB were more likely to respond to several calls but give significantly different firing rates to just one, whereas neurons in area S were more likely to respond only to one call type. Furthermore, a variety of temporal coding firing patterns were observed, such as onset/offset responses or responses to frequency changes within a call. Neurons in VRB, AI and S were also the most likely to give isomorphic responses. That is, the firing pattern would closely match the entire waveform envelope of the sound wave.

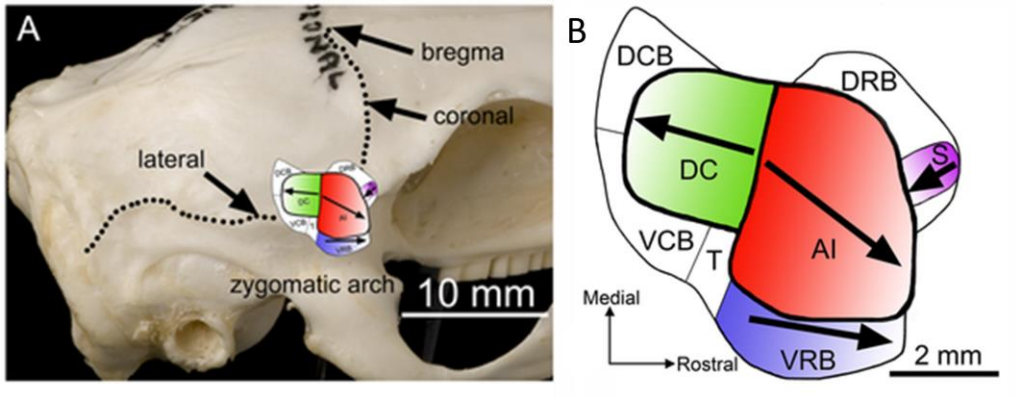



**Figure 1-7** Guinea pig auditory cortex. A) The location of auditory cortex with relation to surface landmarks on the skull. B) The eight defined subregions of auditory cortex. Three of which have a tonotopic gradient. The arrows indicate the direction of high to low frequency. Adapted from Grimsley et al. (2012)

### 1.23 Aims of the combined microstimulation and electrophysiology study

The ability to perform self/external discrimination of auditory information exists across a disparate range of mammalian species. It is likely that this system is well developed in guinea pigs given a) the importance of vocal communication to their survival and b) the discovery of VPL in mice, also a rodent species.

To date, a role for the amygdala in this system has not been considered. This brain region is known to integrate multimodal sensory information (Kuraoka and Nakamura, 2007; Nishijo et al., 1998) and, in human, it is involved in the perception of emotional prosody (Anderson and Phelps, 2001). Anatomical studies in nonhuman primates and rodents have shown connectivity with three auditory structures: IC (Marsh et al., 2002), MGB (Adolphs et al., 2002) and AC (Amaral and Price, 1984; McDonald and Jackson, 1987).

By combining electrophysiological techniques with microstimulation to elicit vocalisations I aim to address these questions:

Does activation of the amygdalar vocal production region modify the representation of conspecific vocalisations in VRB and primary auditory cortex?

Is the response to certain vocalisations selectively enhanced/suppressed more than others?

Does this selectivity correspond with the type of vocalisation that can be elicited from the basal amygdala?

# Methods

## 2.1 Animals

Pigmented guinea pigs (Cavia porcellus) were bred in-house and all procedures were carried out according to UK Home Office regulations. Male and female animals were used in the weight ranges: microstimulation 342 -1348 g, audio-vocal 597 – 840 g.

## 2.2 Single and Dual Microstimulation surgical procedure

Anaesthesia was induced in the guinea pig by an intraperitoneal injection of 20% urethane solution at 2.5 ml/kg body mass and supplemented, as necessary, by intramuscular injections of ketamine/xylazine (3:2 by volume), (Ketaset/Rompun) to abolish the forepaw withdrawal reflex during the surgery. Atropine sulphate was given subcutaneously shortly after the onset of anaesthesia, 0.4ml/kg, concentration 600mg/ml (Hameln pharmaceuticals). Atropine is a parasympathetic depressant which aids the animal's breathing by causing bronchodilation and reducing bronchial secretions.

The head and ears were shaved. An incision to the tragus was made to allow access to the ear canal and the animal was mounted in a stereotaxic headholder. A long midline incision was made over the cranium and the periostium scraped back using a scalpel. The wound was held open with haemostats. The head was levelled at 5 and 13mm anterior to ear bar zero (Rapisarda and Bacchelli, 1977) and the head was fixed to a steel bar bolted to the stereotaxic instrument using stainless steel screws and dental acrylic. Craniotomies and durotomies were made according to the brain region to be stimulated (Rapisarda and Bacchelli, 1977). Once the dental acrylic had set the incisor bar was removed to allow unimpeded jaw movement. The level of

anaesthesia was allowed to become lighter over the next few hours. The level of anaesthesia was constantly monitored and if there were any spontaneous hindpaw movements then supplementary doses of ketamine/xylazine mix were administered (usually in a dose of 0.05 ml). Animals were unable to vocalize while under deep surgical anaesthesia.

**2.3 Surgical procedure for audio-vocal study**

The surgical procedure for the audio-vocal study was the same as for the microstimulation study with the following exceptions: Hypnorm (fentanyl citrate: 0.315 mg/ml and fluanisone: 10 mg/ml) was used in place of ketamine/xylazine which was found to have a detrimental effect on cortical activity. Once the head was secured the two speculae were withdrawn ~1 mm to relieve some of the nociceptive input. Stimulation electrodes were only inserted once per experiment and could be inserted directly through the dura.

Following confirmation of the vocal production area, by stimulating via the implanted electrodes, additional anaesthetic (Hypnorm) was administered. To equalise pressure across the tympanic membrane, polyethylene tubes were inserted into a hole in the ethmoid bone and sealed with petroleum jelly. The posterior fossa was pierced to reduce respiration-related movement of the brain. Ear speculae were pushed firmly against the animal's head and checked to confirm visibility of the tympanic membranes.

### 2.4 Multi electrode arrays

For electrophysiological recording, glass-coated tungsten microelectrodes were produced using in-house equipment as described by (Bullock et al., 1988). Four electrodes (impedance 1-3 MΩ) were attached to a printed circuit board using epoxy resin with approximately 100 – 200 µm between each electrode tip. All electrodes protruded an equal distance from the board.

For electrical microstimulation, lower impedance electrodes (capable of conducting up to 500 µA) were produced in a similar manner with these exceptions: Etching time was reduced by 50 % to give broader, more robust electrodes. More glass (~100µm) was removed from the tips. The four-electrode array was arranged with ~500 µm.

### 2.5 Microstimulation

#### 2.5.1 Microstimulation for the vocal production study

For the microstimulation study a single channel (AM Systems) and a four channel (Multichannel Systems) isolated pulse stimulator were used to deliver biphasic 1 ms square wave pulse trains at 60 Hz for 1.6 s. Both stimulators could be triggered manually and the duration of the train and the frequency of the pulses could both be altered if necessary. The four channel stimulator could also be programmed, as for the dual stimulation study. Here the pulse train described above was delivered to both areas separately (A & B) then simultaneously (C) in the order A B C B A C, with a 10 s gap between each pulse train.

Electrode arrays were aligned in the anterior-posterior plane. When only one array was used, the stereotaxic apparatus was vertical. When two arrays were used, both

stereotaxes were angled 10° lateral to vertical, so as to avoid collision. In reference to cranial landmarks (either ear bar zero or bregma), arrays were positioned above the relevant brain areas. Hydraulic microdrives (Neurocraft) were used to advance the electrodes into the brain.

Positions of stimulating and recording electrodes could be marked with electrolytic lesions created using a 10s monophasic negative square wave pulse at between 10 - 100µA. A minimum of 60 min was left between lesioning and perfusion to allow characteristic necrotic changes to occur in the brain at the lesion site.

### 2.5.2 Microstimulation for the audio-vocal (AV) study

The two previous AV microstimulation studies used square wave trains to activate vocal production loci (Alexander et al., 1976; Müller-Preuss et al., 1980). In both cases, electrophysiological recordings in AC were masked by the artefact of the stimulation, so the researchers were restricted to observing the neural activity in the period immediately following the cessation of stimulation. To counteract this problem, the present study, instead, used continuous 100 Hz sine wave stimulation. Once the neural recording had been filtered (between 600 Hz and 3 kHz), this artefact was sufficiently reduced so as to allow observation of single neuron activity. Continuous sine wave microstimulation has been used by others to activate particular brain areas (Hall and Lindholm, 1974; Tai et al., 2003). There are no reports, however, of this kind of stimulation being used in combination with electrophysiology for the purpose of artefact reduction.

The 4 channel isolated pulse stimulator (Multichannel Systems) was used to deliver 100 Hz continuous sine wave stimulation, oscillating between 100 and -100 µA for

800 ms. In a lightly anaesthetised GP, this was sufficient to elicit a vocalisation. At the deeper level of anaesthesia used for neural recordings, the GP did not vocalise.

### 2.6 Audio recording of vocalisations

The vocalisations produced during the microstimulation experiments were recorded using battery powered Handy Recorder H2 (Zoom) (16 bit, sample rate 96 kHz) placed ~5cm in front of the guinea pig's mouth.

### 2.7 Histological verification of electrode position

#### 2.7.1 Perfusion

To ensure thorough and rapid fixation of the brain, the whole animal was perfused via the heart. Animals were killed by overdose: an intraperitoneal injection of 3ml/kg sodium pentobarbitone (200mg/ml) (Euthetal). Hypoxia causes vasoconstriction and would impede the passage of the fixative. Therefore, the animal was perfused within about 5 min of overdose as soon as the heart had stopped beating.

Approximately 500ml 4% paraformaldehyde fixative was held at a 1.4 m height to achieve 120 mm of mercury pressure (approximately the same as normal systolic pressure) and passed via a tube and needle into the left ventricle.

After perfusion, the animal was decapitated and the brain removed using rongeurs. The brain was stored in a jar of fixative in the fridge until required for sectioning.

### 2.7.2 Gelatine embedding mixture

To assess the location of stimulation electrodes the brain was embedded in gelatine prior to sectioning in order to prevent folding of the sections and to maintain the position of all the brain areas relative to each other.

10g chicken egg albumin (Sigma) was added to 25ml 0.1M phosphate buffer, stirred at room temperature, first with a glass rod, then a magnetic stirrer for 10-20 mins.

0.25g gelatine was dissolved in 25ml 0.1M phosphate buffer using a magnetic stirrer and hotplate at 60°C.

The above two liquids were added together and stirred at room temperature. 10ml was added to a beaker and 0.7 ml of glutaraldehyde (Sigma) was added using a pasteur pipette, stirred with the pipette and added to a mould containing the brain block within 5 sec. Excess gelatine was trimmed off and the block glued to a sectioning chuck using superglue. One corner of the block was cut to facilitate correct orientation on the slide. 100µm sections were cut using a vibratome and placed in wells of phosphate buffer (pH 7.4) prior to mounting on subbed slides.

### 2.7.3 Nissl staining

Cresyl violet stains acidic groups such as those in DNA/RNA within neurons and is useful for observing gross morphology of nuclei and sub-nuclei. Electrolytic lesions show as clear, unstained patches and the electrode tracks can often be seen because they fill up with blood. Retrospective determination of electrode position is more accurate than stereotaxic coordinates and, in case of any mismatch, histological data was taken as definitive. See section 2.9.1 for further details.

Mounted brain sections were submerged overnight in ethanol/chloroform (50:50), then hydrated through a series of graded alcohols (100%, 95%, 70%) for 2 min each, followed by 2 x 2 min washes in distilled $H_2O$, then stained in cresyl violet solution (Sigma) for 8min.

Slides were then washed for 2 x 2 min in distilled $H_2O$ and dehydrated through the same alcohol series then placed in histolene clearing agent (Histoclear) for 5 min before mounting with a coverslip using DePeX mountant (Sigma).

**2.8 Single-neuron recording**

After removing the dura, an electrode array was positioned at $40^0$ to the vertical plane, using cranial landmarks to align with right auditory cortex. This was ipsilateral to the stimulating electrode. Electrodes were connected to TDT System 3 via a TDT Medusa headstage amplifier. The TDT system was controlled using Brainware (software by J. Schnupp, University of Oxford, UK). The array was advanced using a hydraulic microdrive in 10 µm steps. The vocalisation battery was found to be the most effective search stimulus for locating auditory driven cells. Extracellular single-units were filtered between 600 Hz and 3 kHz. Only unambiguous single-units with good signal to noise ratio were analysed.

**2.8.1 Auditory stimuli**

Auditory stimuli were delivered to both ears via earphones inserted into the specula (Etymotic ER-4 earphones, Etymotic Research Inc., IL, USA). A range of 7 vocalisations were chosen from the previous microstimulation study. These had a variety of spectrotemporal structures and also covered a range of motivational state indicators. A control stimulus of broadband noise with the same temporal structure as a ‘whine’

was included. Each sound file was cut from 20 ms prior to onset of vocalisation (see figure 2.1) and normalised using Audition 3.0 (Adobe). 1000 ms of physiological activity was recorded with a gap of the same length between presentations. The eight audio samples were presented both with and without concurrent electrical stimulation. These 16 conditions were randomly presented and repeated 20 times. Microstimulation started at time 0 and lasted for 800 ms. Audio presentation started at time 100 ms.

The $F_0$ of the whine call is sensitive to stimulation current and anaesthesia level (see section 3.2). These same parameters may also affect EC. For this reason, three exemplars of whine, with a range of $F_0$ values, were used. The other audio samples were chosen to represent a range of both emotional meanings and spectrotemporal structures.

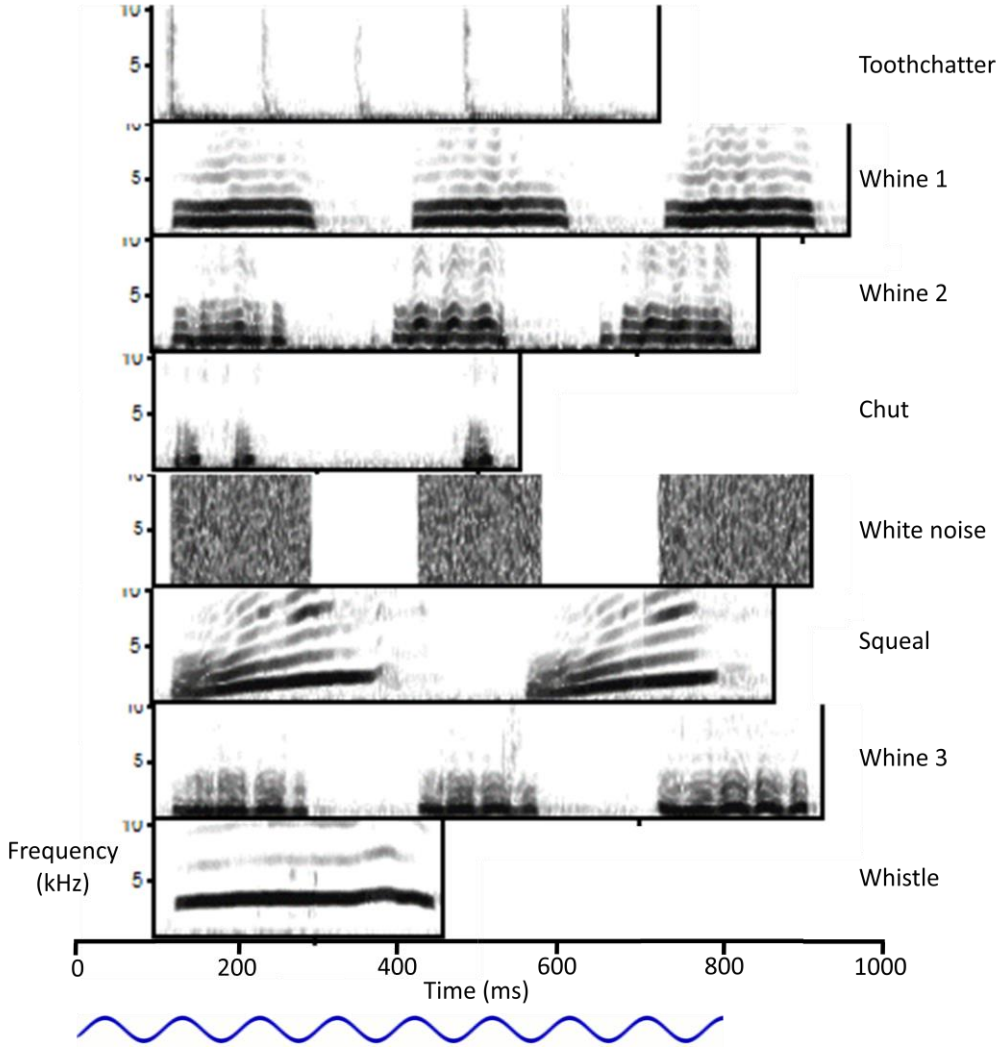



**Figure 2-1** The eight vocalisation samples (including white noise control) were presented with a 100 ms delay. The blue sine wave indicates the electrical stimulation.

In addition to the test described above, each neuron was characterised by its response to broadband clicks and pure tones, by measuring frequency response areas over the range 50 Hz – 25 kHz (50 ms duration, 200 ms repeat rate, 0.25 octave intervals, sound levels of 0 – 100 dB in 5 dB increments from a maximum 100 dB SPL).

## 2.9 Data Analysis

### 2.9.1 Mapping of vocal production areas

The animals used in the microstimulation experiments covered a broad size/weight range. To account for this, coronal sections were mapped in the anterior/posterior direction to a standardised guinea pig atlas (Rapisarda and Bacchelli, 1977) using landmarks dispersed across the whole section in addition to the brain region of interest.

Images of Nissl stained slides were created using an Axioskop 2plus transmitted light microscope with motorised stage (Carl Zeiss), connected to a PC with Neurolucida software version 10 (MBF bioscience). Only a portion of a brain slice could be imaged at one time. Automated image stitching software (PanoramaPlusX4, Serif) was used to create a single image of a whole brain slice.

Vocal production loci were distributed across cerebral hemispheres. For clarity, and to allow one to read the anatomical labels, all loci were transcribed to the left hemisphere. Figure 2-2 shows example Nissl stained coronal sections with electrolytic lesions.

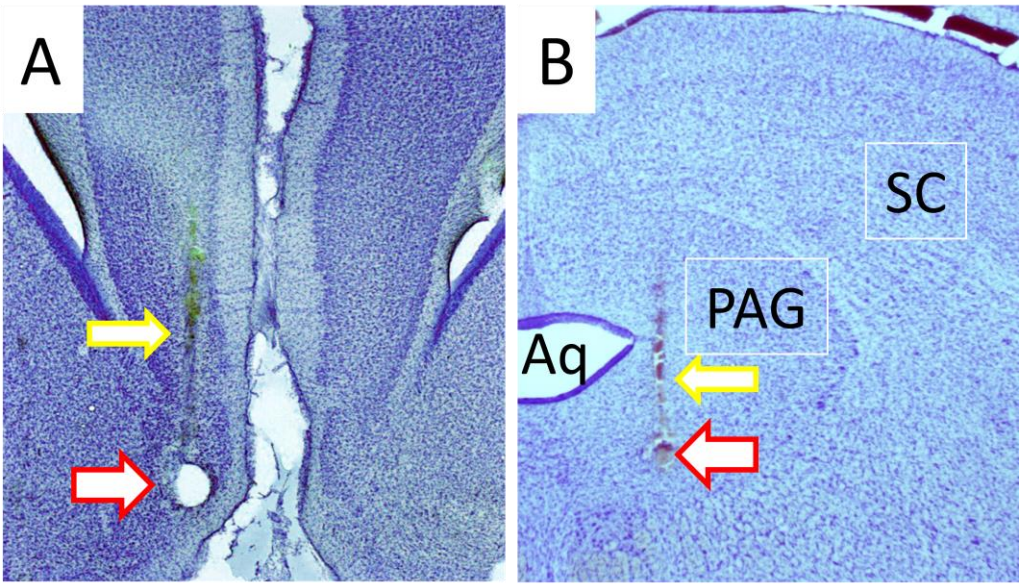



**Figure 2-2** Nissl strained coronal sections in ACC (A) and PAG (B). Red arrows indicate electrolytic lesions, yellow arrows indicate track marks. Other abbreviations: Aq, cerebral aqueduct, SC superior colliculus.

### 2.9.2 Characterisation of vocalisations

Audio recordings of spontaneously vocalising guinea pigs were provided by Prof. J Grimsley. These, and the electrically elicited calls, were analysed using the same procedure.

SAS Lab Pro bioacoustics software (Avisoft) was used to automatically detect the waveforms of individual vocalisation sound pulses (figure 2-3A), and $F_0$ data was extracted at 1 ms intervals (figure 2-3B). Occasionally, the software would incorrectly label a harmonic as the $F_0$. By using this high sample rate, the effects of these errors was rendered inconsequential. Matlab (Mathworks) was used to perform linear regression of the $F_0$ values for each sound pulse. The data for starting $F_0$ and $F_0$ gradient for each sound pulse were extracted from this regression.

The four classification parameters were: duration of sound pulse, sound pulse frequency, $F_0$ at the start of a sound pulse and gradient of $F_0$. These parameters could not be assigned to all call types. For example, the whistle call is only delivered in a single sound pulse and cannot be assigned a value for pulse frequency.

The ambient, low frequency noise from the air conditioning system in the laboratory is visible on all spectrograms. The $F_0$ values of some calls were close to this noise, preventing automated analysis. When necessary, visual inspection of the spectrograms was used to gain these data.

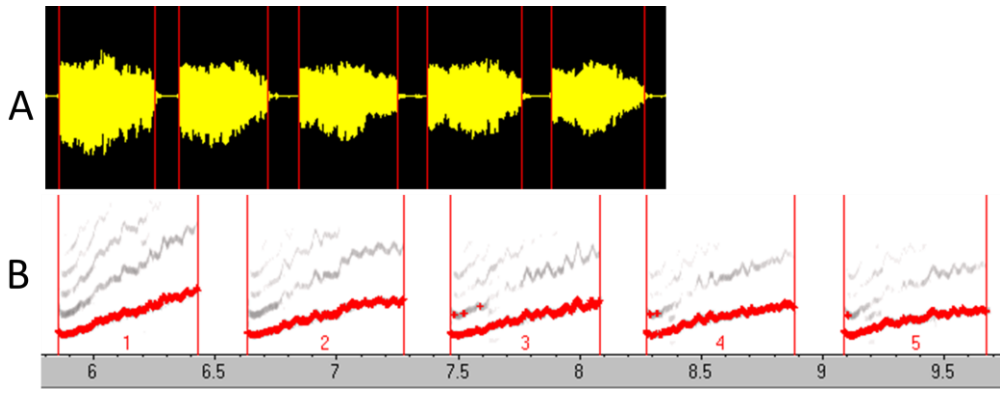



**Figure 2-3** A) Labelling the sound pulses from one call bout based on the wave form amplitude. B) Data extraction from the pre-defined labels. $F_0$, highlighted in red, was sampled every 1 ms.

The electrically elicited calls had distinct spectrotemporal characteristics, allowing them to be separated, hierarchically, into eight distinct classes. They were named according to Berryman (1976) and Grimsley (2011). In all but one instance the classification of the electrically elicited call was unambiguous. This is discussed in section 3.

### 2.9.3 Dual stimulation analysis

Three types of simultaneous dual stimulation were performed on combinations of brain areas: hypothalamus, amygdala, anterior cingulate cortex, thalamus, caudate putamen, medial forebrain bundle and lateral septum.

The spectrotemporal structure of vocalisations resulting from each individual area and with dual stimulation were assessed using the system described above. In all cases these vocalisations were within the parameters described for each call type in the first microstimulation investigation (see section 3).

Root mean squared (RMS) values of the waveform envelope were calculated for the post-stimulus time window (2 – 10 s) using Audition 3.0 (Adobe). Wilcoxon signed-rank test (SPSS, IBM) was used to compare the dual stimulation data with those of each individually stimulated area.

In a similar manner, RMS values were used to compare the time course of amplitude modulation of during- and post-stimulus vocalisations. These data were extracted for two time windows of 1.6 s: the during-stimulus window extending backwards from the end of the call (to account for offset latency), and the post-stimulus window extending forwards from the onset of the first sound pulse.

### 2.9.4 Audio-vocal analysis

For each vocalisation a normalised response modulation index (RMI) was calculated between the electrically stimulated and unstimulated conditions (Eliades and Wang, 2003). The spike counts for the whole 1000 ms sweep were summed across the 20 repetitions in the unstimulated, and 20 repetitions in the electrically stimulated

conditions. RMI values were calculated using the following formula:

$RMI = (Spikes_{Electrical\ Stim} - Spikes_{No\ Stim}) / (Spikes_{Electrical\ Stim} + Spikes_{No\ Stim})$

i.e., the effect of electrical stimulation with respect to the unstimulated condition.

The significance of these RMI values was determined by creating randomised data sets to ascertain the magnitude of RMI that could be expected by random variation. An illustration of the process is shown in figure 2-4 with the neural responses to two vocalisations in the same neuron. A and C show post stimulus time histograms of the neural response to chut and whine, respectively. The blue histograms show the responses to vocalisation alone, the green histograms show the response to auditory and electrical stimulation. The 40 spike count values for each vocalisation response (20 unstimulated and 20 stimulated) were randomly permuted, split into two sets of 20 values, and then summed. These values were used to calculate an RMI value using the above formula. This process was repeated 1000 times and the 95% intervals of the distribution were calculated. The histograms in B and D show these distributions (for A and C, respectively) of the randomised RMI values, with the 95% confidence intervals shown by the red dashed lines. RMI values calculated from the original data set are shown by the green line. Only RMI values that fell outside the confidence intervals were deeded significant.

All the analyses described above were performed using Matlab (Mathworks).

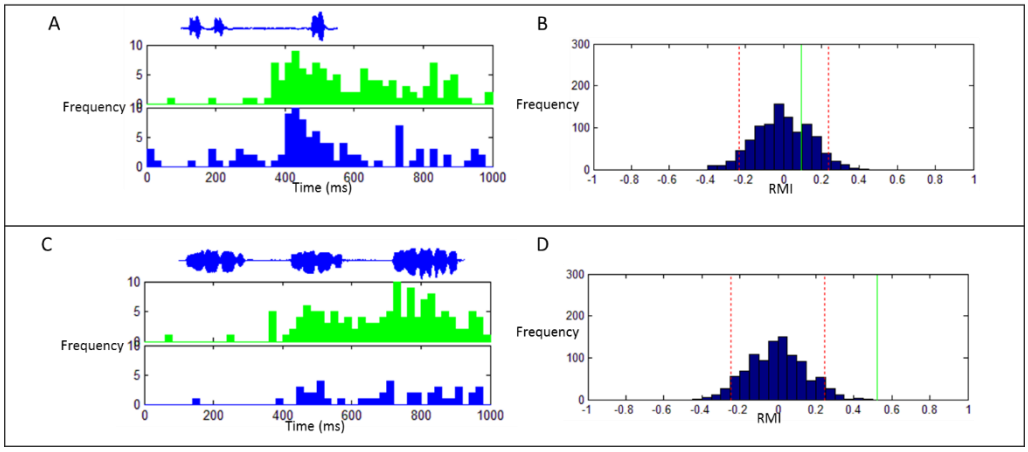



**Figure 2-4** An illustration of the RMI significance testing method. A and C show post stimulus time histograms of the responses to chut and whine, respectively, in the same neuron. The vocalisation wave form is shown above each. Blue plots show responses to auditory-only stimulation, green plots show combined auditory and electrical stimulation (bin size: 20 ms). Histograms B and D show the distributions of randomised RMI values for A and C, respectively (bin size: 0.05 RMI). The red dashed lines show the 95% confidence intervals. The green lines show the RMI values calculated from the original data sets. Therefore, the response modulation by electrical stimulation was deemed significant in D but not B.

# Eliciting Vocalisations by Electrical Microstimulation

## 3.1 Call types produced

The calls elicited using microstimulation were classified in a hierarchical fashion into eight distinct types. These were then named by comparing them to spontaneously produced calls from the same colony (Grimsley, 2008). Six of the electrically elicited call types could be unambiguously paired with their spontaneous counterpart. The only exception, chutter, will be discussed in due course. Example spectrograms of individual sound pulses are shown in figure 3-1.

In total, 61 animals were used for these experiments. For stimulation in amygdala, 42 animals were used; for ACC, 12 animals were used; for PAG, 8 animals were used; for hypothalamus, 19 animals were used.

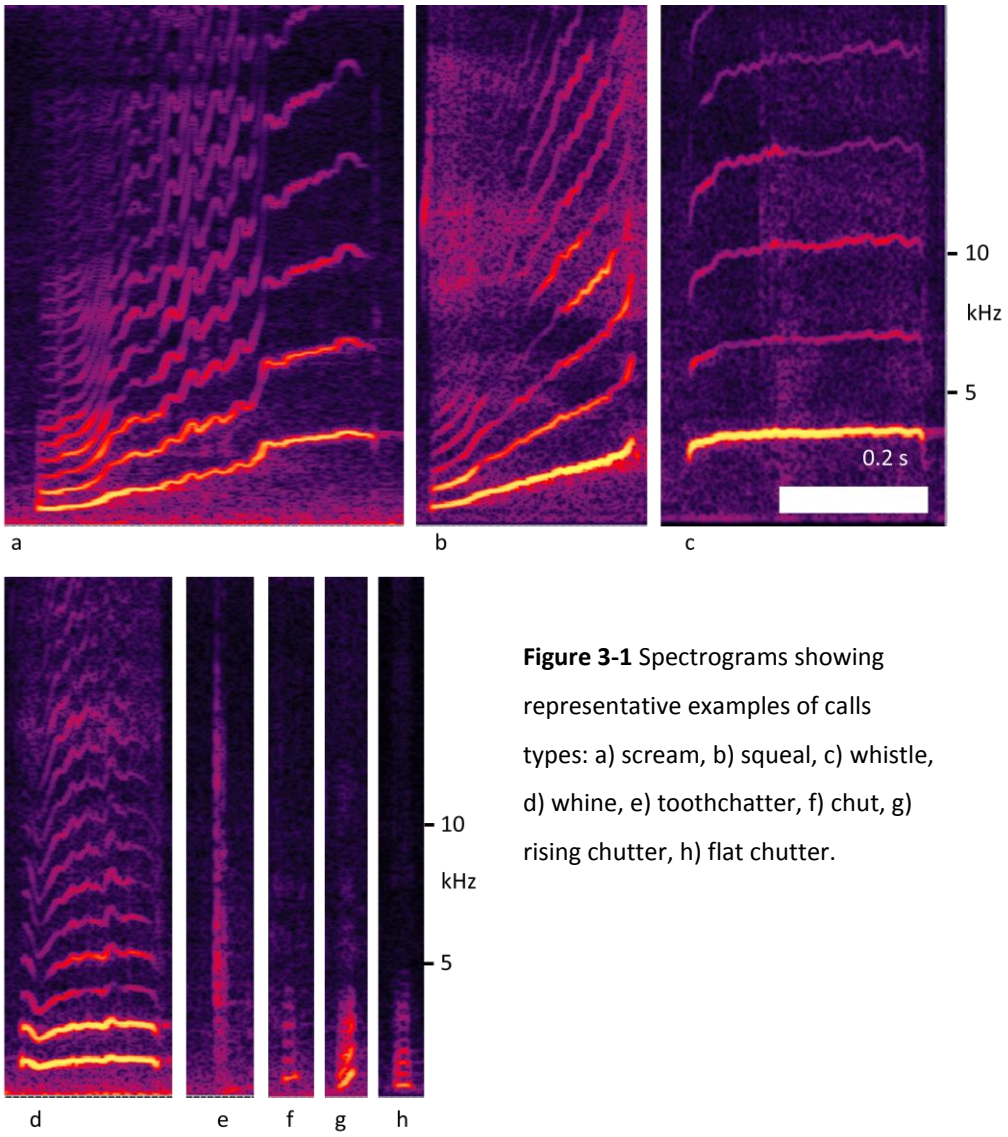



**Figure 3-1** Spectrograms showing representative examples of calls types: a) scream, b) squeal, c) whistle, d) whine, e) toothchatter, f) chut, g) rising chutter, h) flat chutter.

### 3.1.1 During-stimulus and post-stimulus vocalisations

Whine, chut, rising chutter, flat chutter and whistle were produced during the 1.6 s stimulus train. Latency of onset varied from 300 to 800 ms and vocalisation ceased ~150 ms after the end of the stimulus train. In contrast, scream, squeal and toothchatter were produced shortly after (~400 ms) the end of the stimulus train and would last for between 5 – 60 s. All the call types produced during stimulation were never produced post stimulation, and vice versa. Stimulation at a given location could

produce: 1) only a during-stimulus call, 2) only a post-stimulus call, or 3) a during-stimulus call followed by a post-stimulus call.

### 3.1.2 Factors affecting the proportions of call types available for analysis

In the current study certain call types were elicited more frequently than others; presumably for neurobiological reasons. The spontaneous vocalisations to which they were compared were also unevenly distributed, though for social reasons. Animal husbandry in the UK aims to minimise distress to the guinea pigs, so aversive call types such as scream, squeal and whine were rarely recorded (Grimsley, 2008). Previous studies used greater variety of social groups or studied large, semi-wild colonies. In both these cases, aggressive encounters between adult males were common, hence the greater number of related call types (Arvola, 1974; Berryman, 1976).

### 3.1.3 Toothchatter

Toothchatter (TC) is a non-voiced vocalisation. Rapid jaw movements cause the teeth to strike together, producing a series of broadband clicks (Arvola, 1974). This distinctive spectrotemporal structure, shown in figure 3-3, allowed it to be removed from the sample pool in the first instance. TC was elicited from the amygdala in 5 animals and from the hypothalamus in 3 animals. Anatomical locations are shown in figure 3-35. TC is an extremely labile vocalisation; in all but one locus the call could be elicited only once. The only meaningful spectrotemporal property is pulse period. Figure 3-2 shows the analysis of samples from 10 loci (7 in amygdala, 3 in hypothalamus). Bouts of TC were always post-stimulus and ranged from 1 – 8 s. The

ranges of pulse period are similar in both amygdala and hypothalamus. Although no audio samples of spontaneously produced TC were provided, Grimsley stated that TC had a pulse period range of 0.06 – 0.11 s (Grimsley, 2008), meaning that, whilst similar, the electrically elicited TC have a greater variability, with a range of 0.02 – 0.37. An example spectrogram of TC from an awake, spontaneously vocalising animal is shown in figure 3-4.

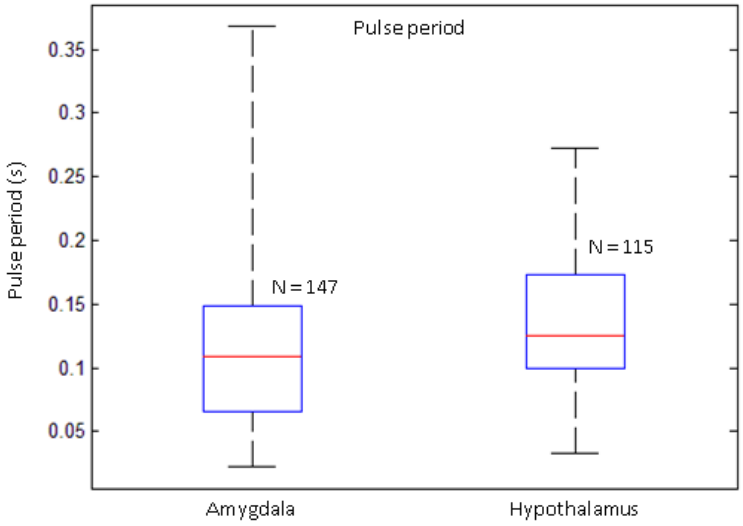



**Figure 3-2** Summarised analysis of electrically elicited toothchatter. N values indicate the number of individual sound pulses that were analysed.

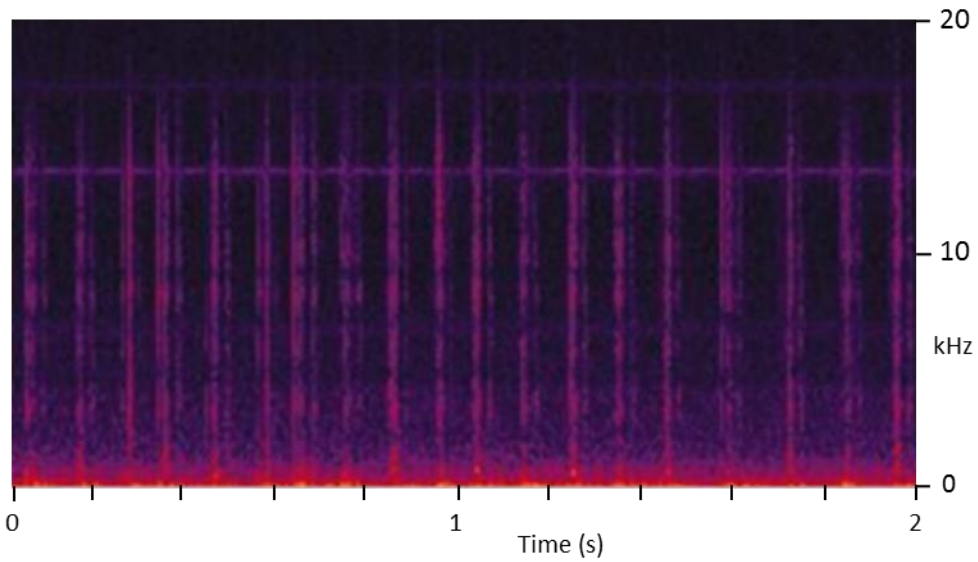



**Figure 3-3** Example spectrogram of toothchatter elicited from the amygdala.

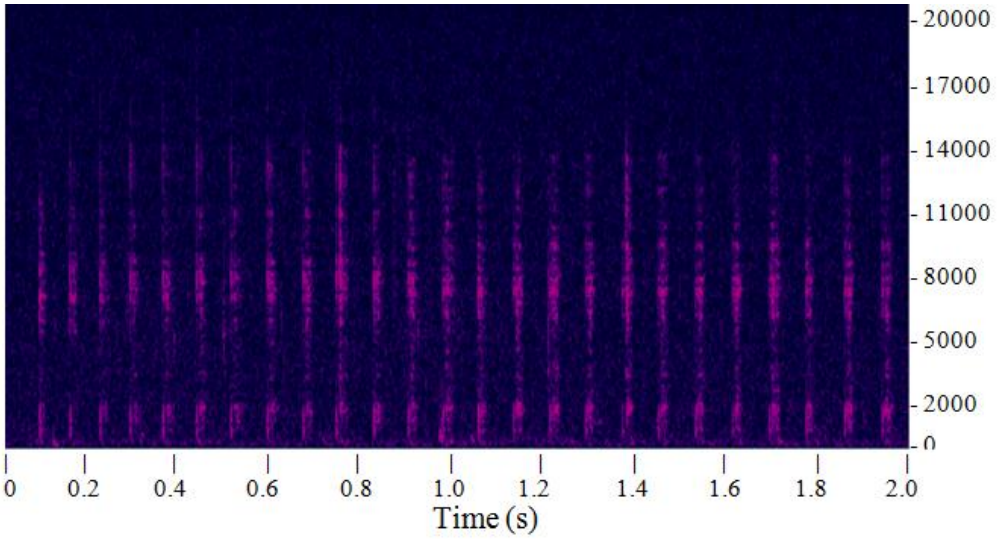



**Figure 3-4** Spectrogram of toothchatter from an awake, spontaneously vocalising guinea pig (Grimsley, 2008).

### 3.1.4 Division of vocalisation type based on duration of sound pulse

The remaining call types could be clearly divided into short and long calls. Figure 3-5 shows a histogram of the duration of all the remaining vocalisation samples, once TC samples were removed. There are two clear populations, with the long calls showing

greater variability. The short calls are: Flat chutter, rising chutter and chut. The long calls are: whine, whistle, scream and squeal.

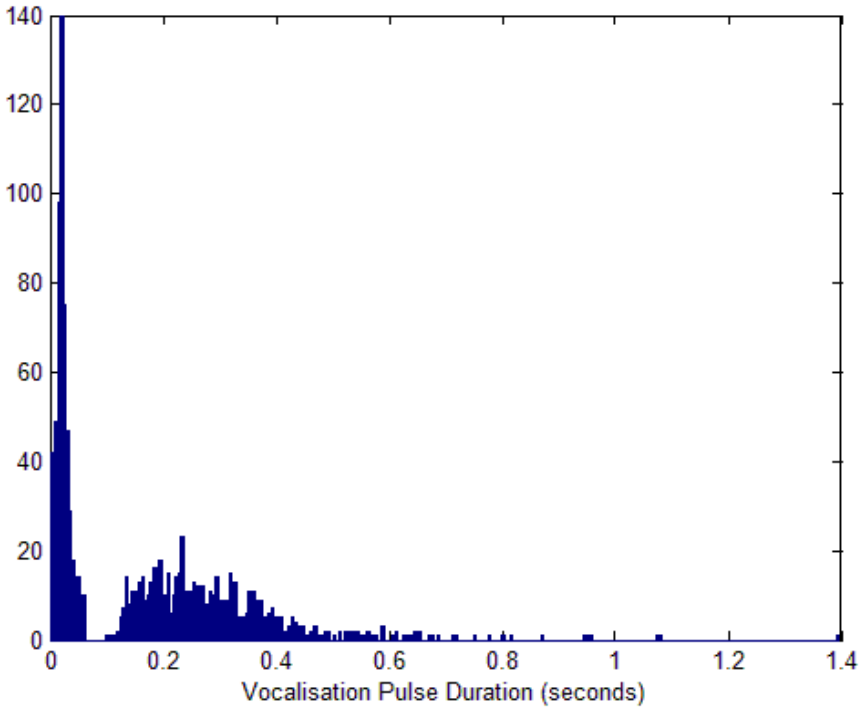



**Figure 3-5** Histogram of 1199 vocalisation pulses (not including TC). Bin width: 5 ms.

### 3.1.5 Chutter

Chutter is the only call for which there was ambiguity. Two of the electrically elicited call types match the spontaneous chutter in terms of duration, starting $F_0$, and pulse period. In terms of gradient of $F_0$, however, the spontaneous call shows variety throughout the call series, whereas the elicited calls fall into two distinct categories: one, named flat chutter, had consistently flat gradient, and another, named rising chutter, which had a positive gradient that increased throughout the call series.

One bout of spontaneously produced chutter was provided by Grimsley. These data are shown in figure 3-6, with a spectrogram shown in figure 3-7.

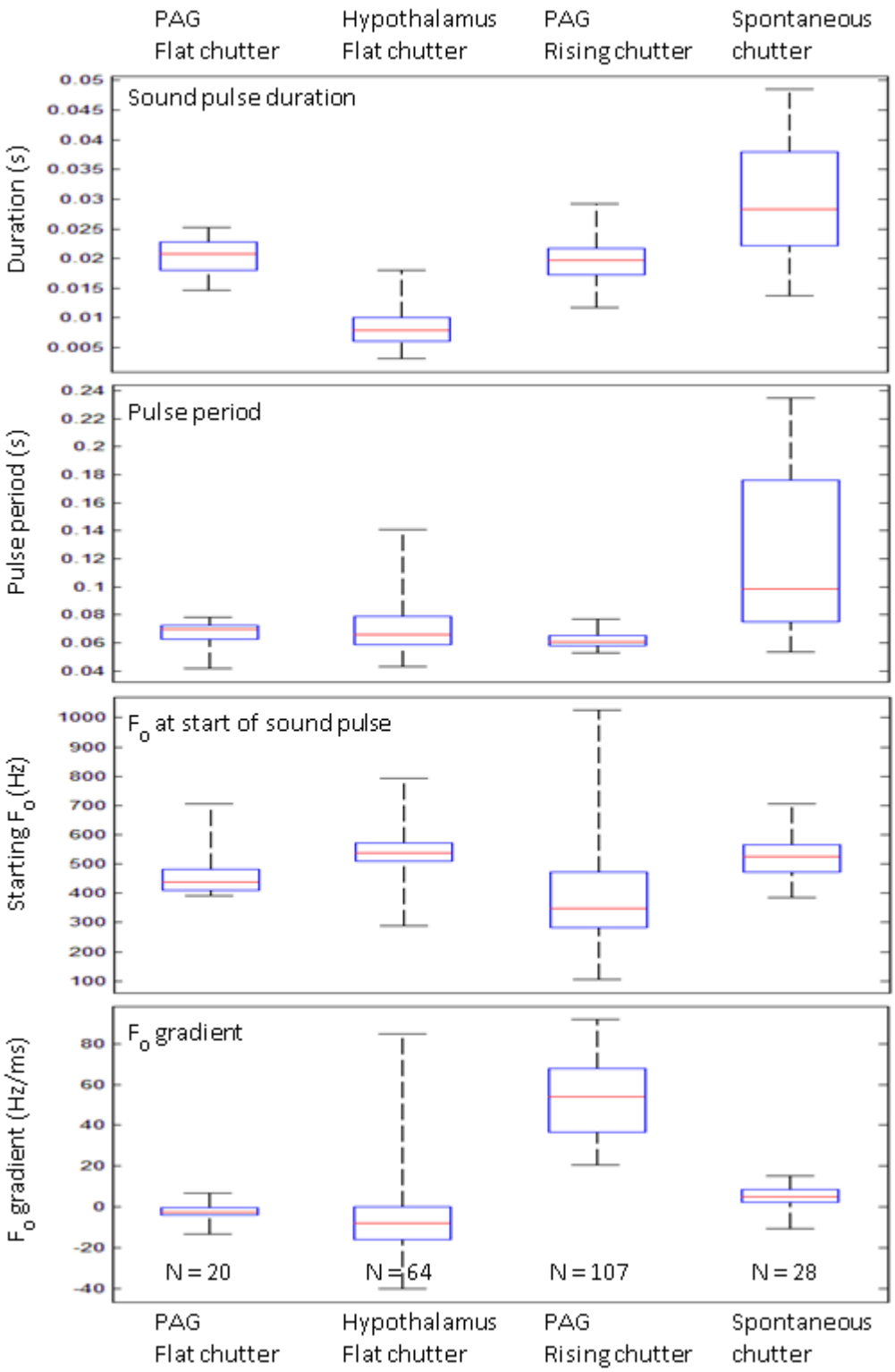



**Figure 3-6** Summarised analyses of electrically elicited flat and rising chutters, and spontaneously produced chutter. N values indicate the number of individual sound pulses that were analysed.

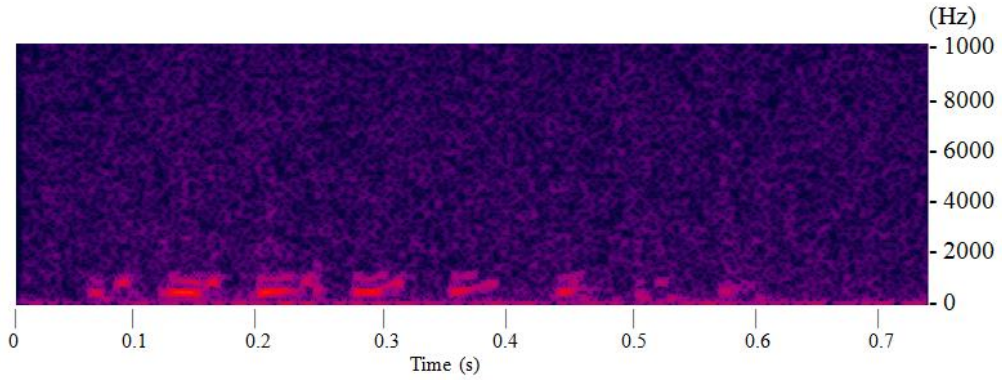



**Figure 3-7** A spectrogram of chutter recorded from an awake, spontaneously vocalising animal (Grimsley, 2008).

Flat chutter was elicited from 5 adjacent loci in the hypothalamus of 1 animal, and from 1 locus in the PAG of 1 animal. Figure 3-6 shows the results from hypothalamus stimulations in four different loci, and 2 PAG stimulations in the same locus. A representative spectrogram is shown in figure 3-8. For all four parameters, there is considerable overlap between the ranges of PAG and hypothalamus that elicited flat chutters. The broader ranges for the hypothalamus are likely an effect of the higher number of samples. The gradient of the individual call pulses showed little variation over the course of a stimulus train but would not progress in a single direction. This is in contrast with the rising chutter, described below.

When compared to the spontaneous chutter, electrically elicited flat chutter from both brain areas share a very similar range of starting $F_0$ and pulse period. The hypothalamic calls have shorter duration calls than the spontaneous calls, though with some overlap in their range. The PAG elicited calls have a similar minimum call length but their range does not reach that of the spontaneous calls. Between the three, all have similar mean gradient, with the PAG and spontaneous calls having similar range and the hypothalamic calls having a larger range.

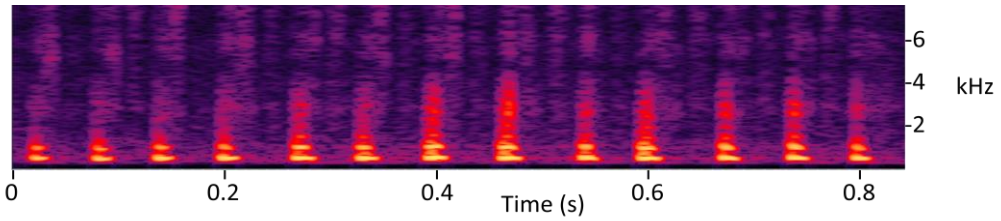



**Figure 3-8** Example spectrogram of electrically elicited flat chutter from PAG.

Rising chutter was elicited from the PAG in three animals. Figure 3-6 shows the summarised analyses, with a representative spectrogram shown in figure 3-9. This call has similar ranges of duration and pulse period to the flat chutter. When single sound pulses are considered, there is also substantial overlap in the range of starting $F_0$. Unlike the flat chutter, however, the gradient was always positive and, over the course of the stimulus train, the rising chutter would increase its $F_0$ and gradient.

In comparison with spontaneous chutter: the duration, pulse period and starting $F_0$ have overlapping ranges. The ranges of gradient, however, are close but non-overlapping. This aspect makes the electrically elicited rising chutter differ from the spontaneous chutter. Though, of the whole GP repertoire, there is no other call that rising chutter resembles.

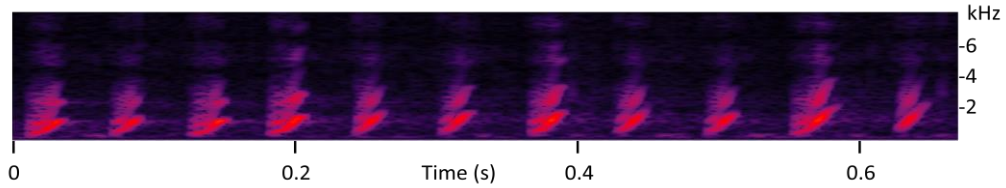



**Figure 3-9** An example spectrogram of rising chutter elicited from PAG.

There are clearly features of both types of electrically elicited chutters which do not match the spontaneous call. The properties they both share with the spontaneous call lead to their being named as chutter. The two subclasses defined above were elicited from adjacent loci in the PAG of one animal (see figure 3-38-B). This raises

the possibility that, in a spontaneously vocalising animal, these loci cooperate to produce the natural sounding call.

### 3.1.6 Chut

The individual sound pulses of the chut call are very similar to those of the chutter. The distinguishing feature is the rate at which the sound pulses are produced. The overall rate is much slower and is also very irregular, ranging from 0.05 – 1.62. Chut was elicited from ACC in four animals, the hypothalamus in six animals and the PAG in four animals. Vocalisations from all three areas showed similar characteristics in all four parameters, as shown in figure 3-10. A representative spectrogram is shown in figure 3-11.

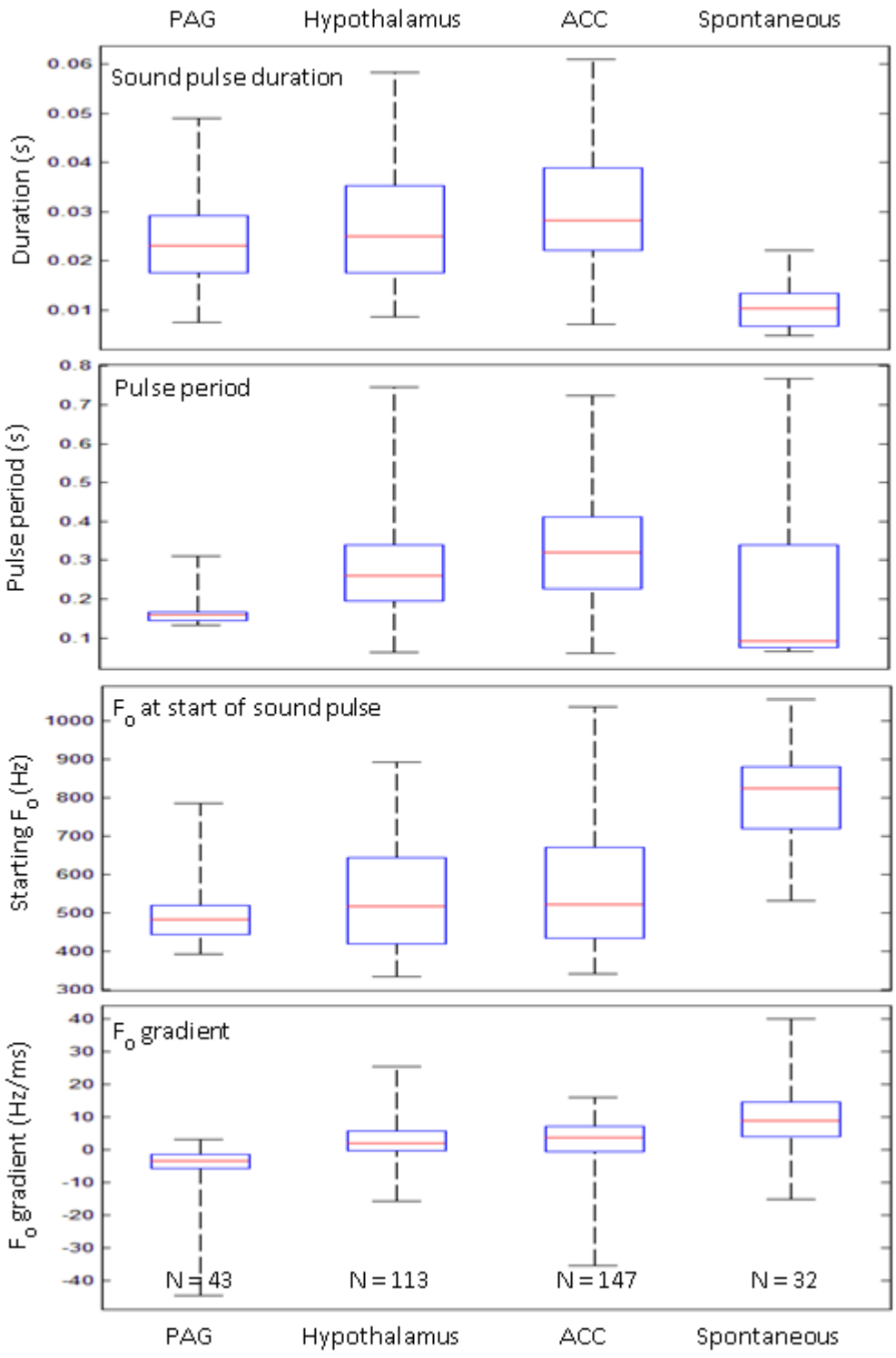



**Figure 3-10** Analysis of chut elicited from three brain areas and spontaneous chut. N values indicate the number of individual sound pulses that were analysed.

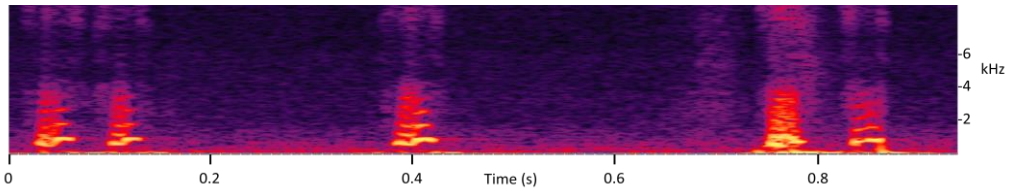



**Figure 3-11** An example spectrogram of chut elicited from the ACC.

Four exemplars of spontaneously produced chut were analysed. These data are summarised in figure 3-10, and an example spectrogram is shown in figure 3-12. For all parameters there is a high similarity between the electrically elicited and spontaneous calls. Of particular note is that the irregular pulse rate is preserved between the groups. This is in contrast to the chutter call, which had an artificially regular pulse rate when electrically elicited.

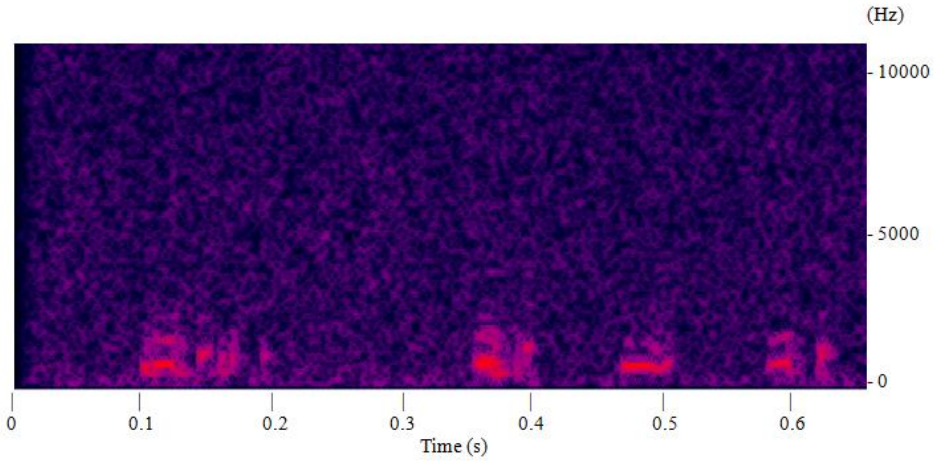



**Figure 3-12** Example spectrogram of a chut vocalisation from a spontaneously vocalising guinea pig (Grimsley, 2008).

### 3.1.7 Classification of the short duration calls

Figure 3-13 allows comparison between all the short duration call types (see figure 3-5). The three electrically elicited short calls – flat chutter, rising chutter and chut – cannot be definitively classified on the basis on the structure of single sound pulses because there is considerable overlap in all parameters. This is expected, since chut

and chutter exemplars from spontaneous GPs cannot be identified from individual sound pulses (Berryman, 1976; Grimsley, 2008). Therefore, the pulse period is necessary to discriminate chut from the two types of chutter: while the chutters never go higher than 0.2 s, the chut exemplars extend to over 0.7 s. The lower range of pulse period is similar in all these call types, highlighting the need to examine the rhythmic or arrhythmic properties of sound pulses within a bout of calls before definitive classification can be performed.

The chutter subclasses can be discriminated on the basis of $F_0$ gradient: the rising chutter has only positive gradient values in this parameter. In contrast the flat chutter has a broad range of negative and positive gradients, distributed relatively evenly around zero. For this reason, several exemplars would need to be examined before a satisfactory classification could be performed.

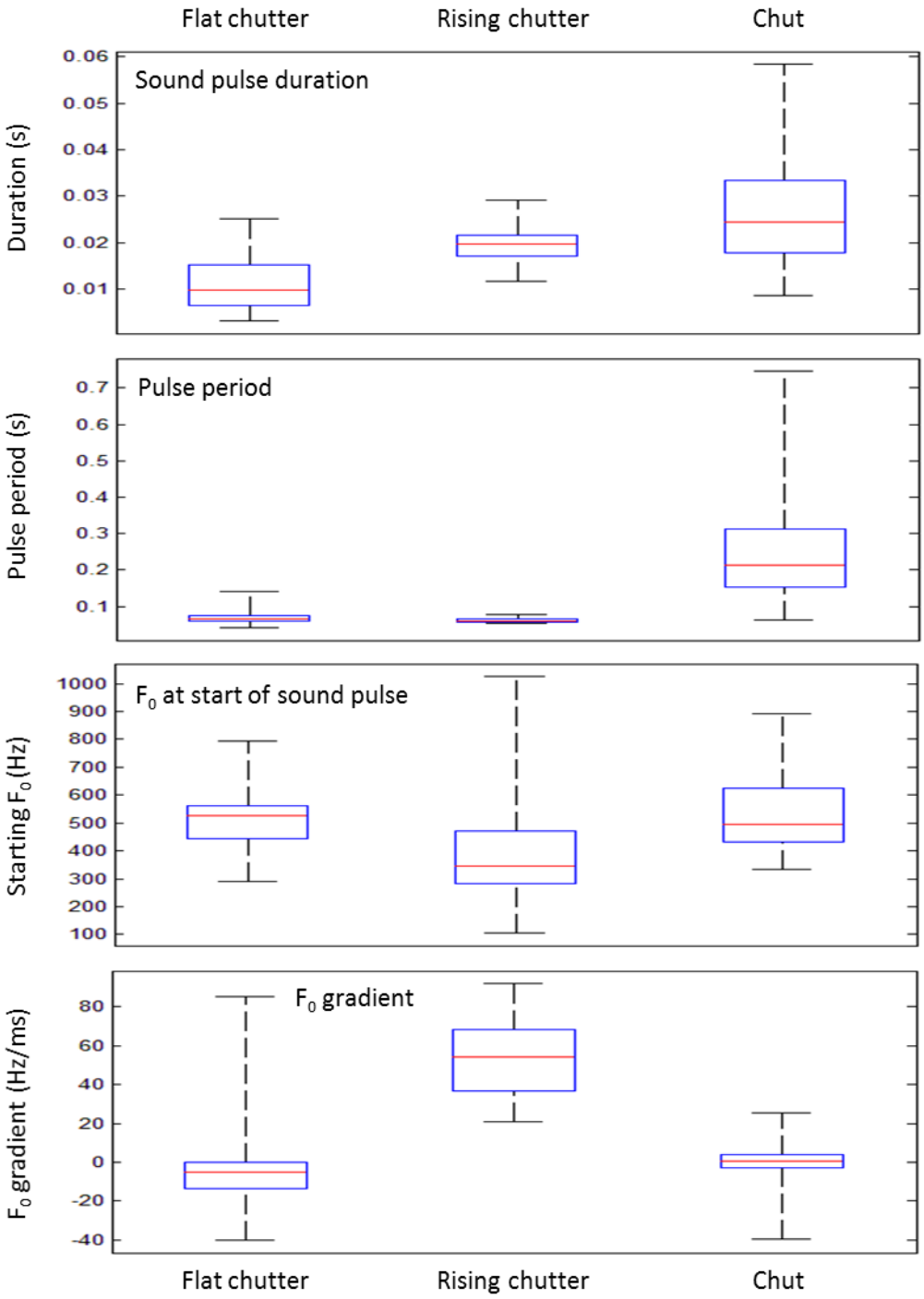



**Figure 3-13** Comparison of the three electrically elicited short duration calls. Recordings from all brain areas, grouped by call type.

### 3.1.8 Whistle

Whistle was elicited from the PAG in two animals, one locus in each with 8 examples recorded. The summarised analyses of these calls are shown in figure 3-13 and a representative spectrogram is shown in figure 3-14. Whilst there is some overlap in the range of starting $F_0$ between whistle and scream the distinct difference in gradient at this $F_0$ level clearly distinguishes them.

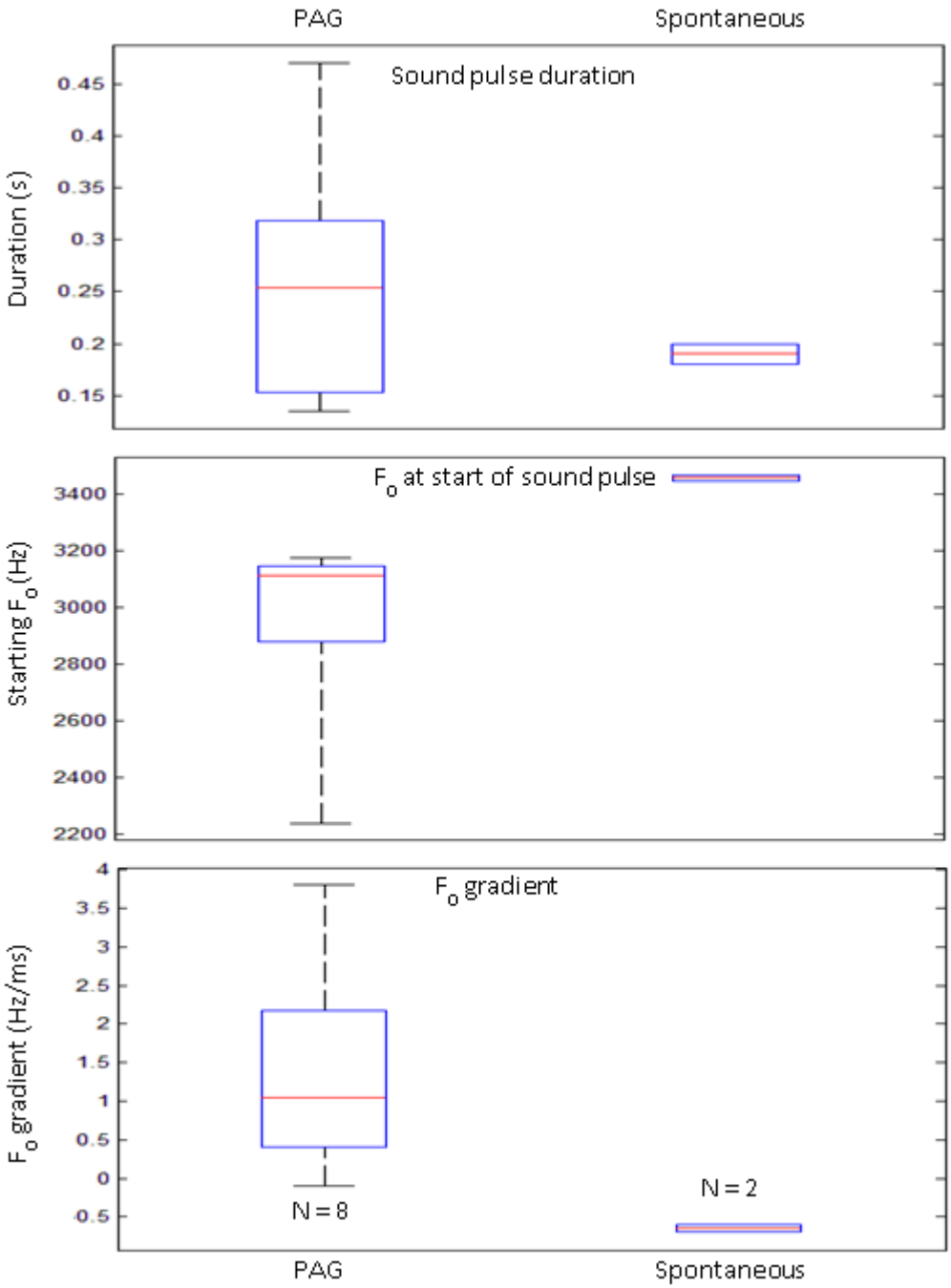



**Figure 3-14** Analysis of electrically elicited and spontaneous whistles. N values indicate the number of individual sound pulses that were analysed.

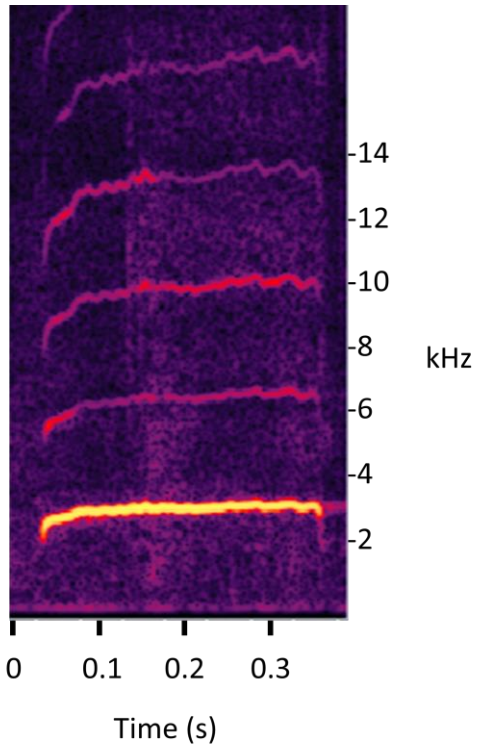



**Figure 3-15** An example spectrogram of an electrically elicited whistle from PAG.

While electrically elicited whistles were only produced in single pulses, the spontaneously vocalising animals gave both single sound pulses (figure 3-15) as well as rhythmic call series (figure 3-16). The sound file for the call series shown in figure 3-16 was not provided; visual inspection of the figure indicates that the individual sound pulses are similar to the electrically elicited ones. Analysis of the two sound pulses shown in figure 3-15 shows that for pulse duration and gradient the structure of the electrically elicited whistles agree with those from spontaneously vocalising animals. The $F_0$s of the spontaneous whistles are higher than the maximum of the electrically elicited whistles yet is within two standard deviations of the mean, and also within the ranges reported by Berryman (1976).

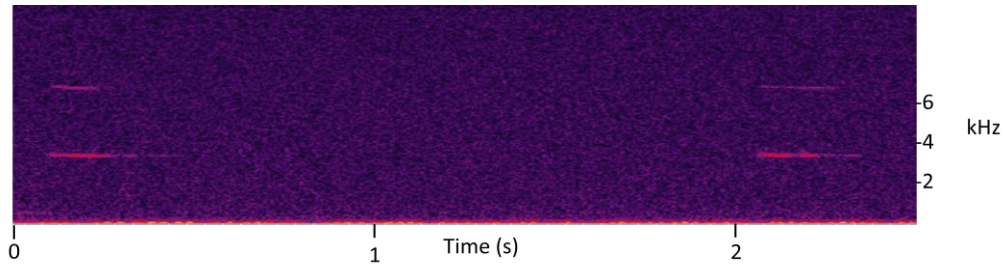



**Figure 3-16** An example spectrogram of two whistle pulses that were not part of a call series. Spectrogram made from original .wav sound file.

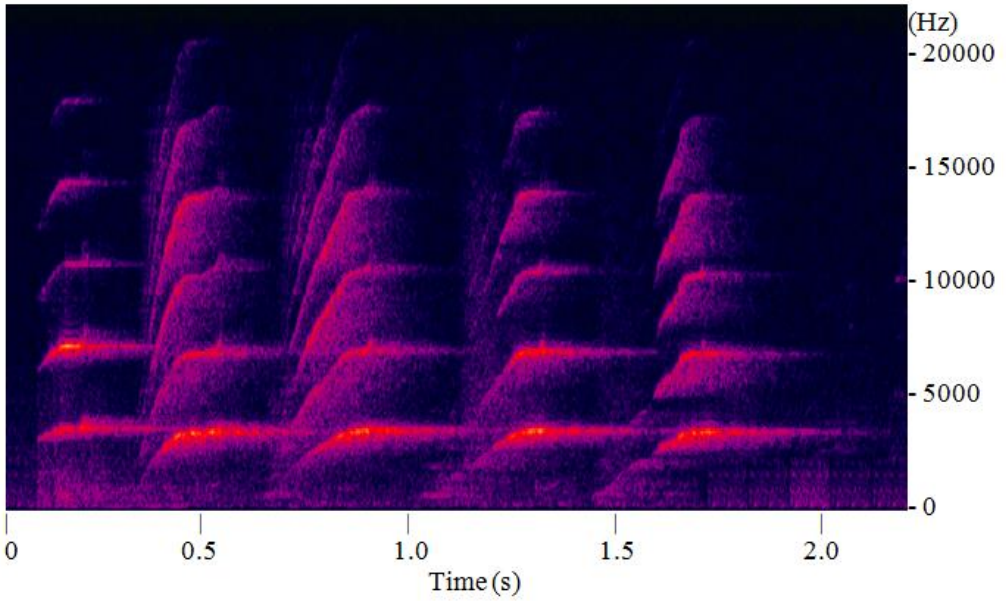



**Figure 3-17** An example spectrogram of a rhythmic whistle call series from spontaneously vocalising guinea pig (Grimsley, 2008).

### 3.1.9 Whine

Whine was elicited from the PAG in six animals, the hypothalamus in one animal, and the amygdala in forty animals. The seemingly disproportionate amount of data from the amygdala is because this brain area was extremely reliable; yielding vocalisations every time it was attempted. In addition, every successful audio-vocal experiment, as well as the unsuccessful troubleshooting trials, yielded whine calls. This call is distinct

from other long calls in that it has a flat gradient yet occupies a lower frequency range than the whistle.

The summarised analyses are shown in figure 3-17, with a representative example shown in figure 3-18. Given that the $F_0$ and duration of the whine vocalisation is sensitive to stimulation current (see section 3.2.1) the distribution of $F_0$ values in figure 3-17 are likely to reflect this experimental variable rather than any biological differences between the three brain regions. Of most importance is that the ranges of the small number of calls elicited from the hypothalamus and the PAG fit within the ranges, for all parameters, of those elicited from the amygdala.

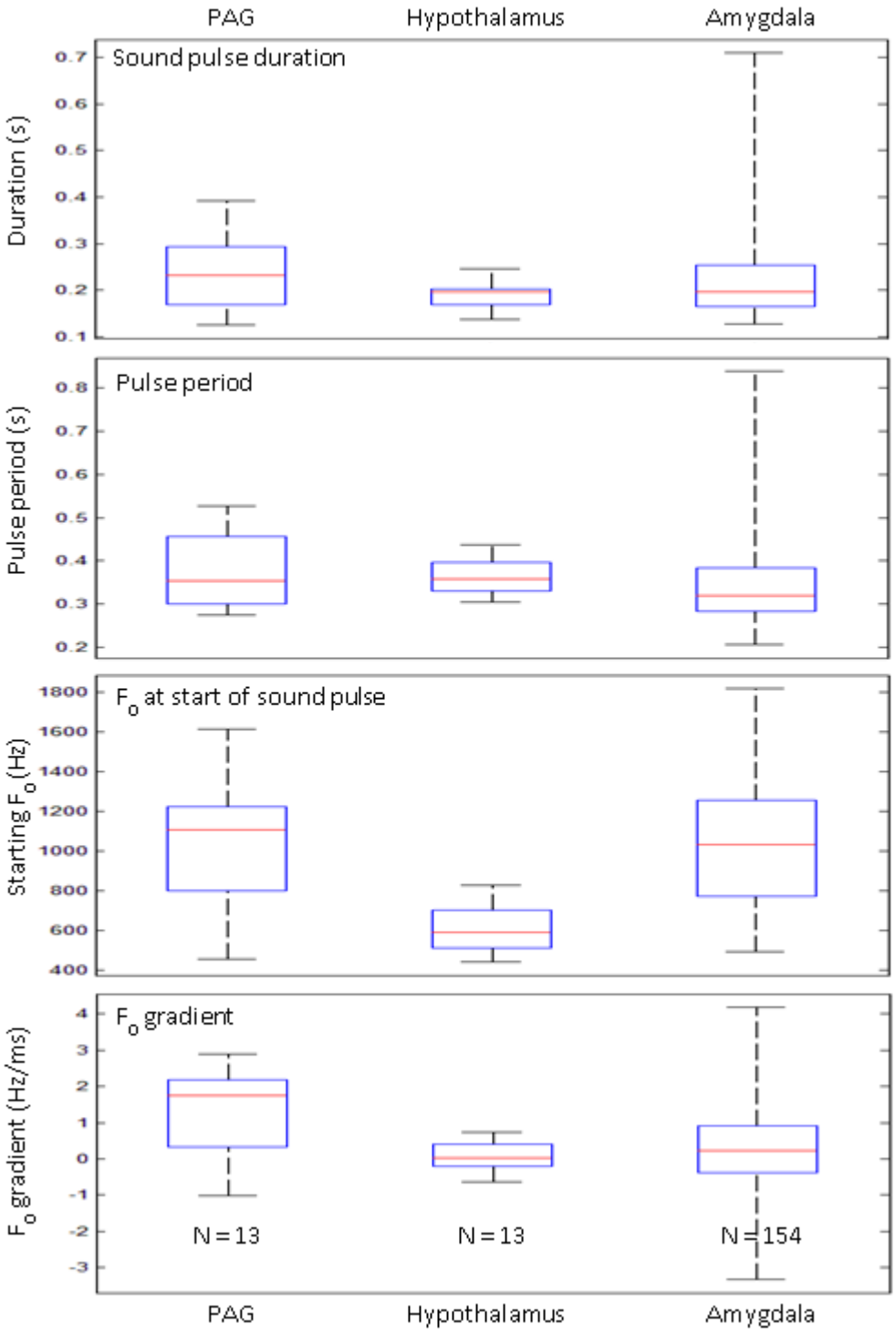



**Figure 3-18** Summarised analyses of electrically elicited whines from three brain areas. N values indicate the number of individual sound pulses that were analysed.

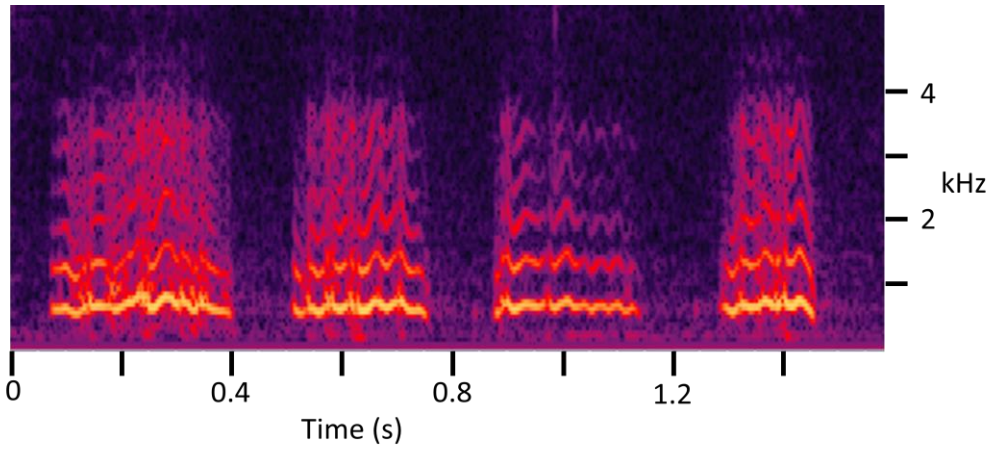



**Figure 3-19** Example spectrogram of a whine elicited from the amygdala.

The whine calls provided by Grimsley were only produced in single pulses, though have been reported to occur in series by Berryman (1976). Of three examples, all had a low $F_0$ that prevented automated analysis. From observation of the spectrograms they had $F_0$ values between 400 and 600 Hz, sound pulse durations between 0.4 – 0.5 s, a flat gradient with undulations (see spectrogram in figure 3-19). These values are within the ranges of the electrically elicited whines. Spontaneous whines with $F_0$ ranges up to 1.6 kHz have been reported in spontaneously vocalising animals (Berryman, 1976).

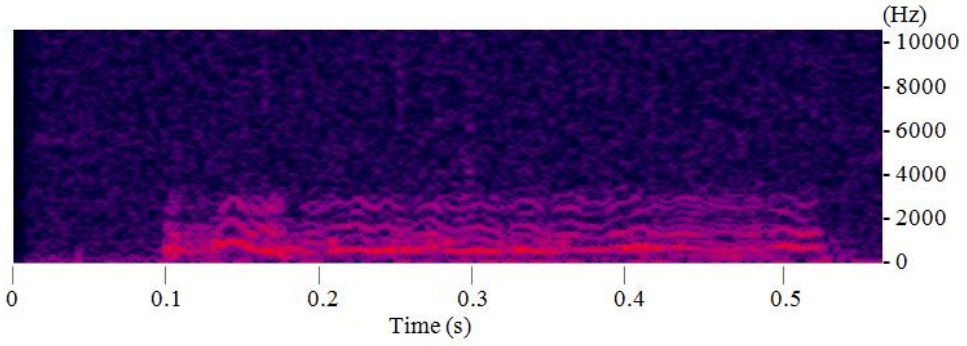



**Figure 3-20** An example spectrogram of whine from an awake animal (Grimsley, 2008).

### 3.1.10 Squeal

Squeals were, by far, the most commonly produced vocalisation. They were elicited from every experimental animal, and production loci were numerous and widespread across many brain regions. Squeal series were elicited from the ACC in twelve animals, the hypothalamus in nineteen animals, the amygdala in eleven animals, the PAG in seven animals and various thalamic nuclei in 13 animals.

These calls were post-stimulus and are sensitive to stimulus current (see section 3.2.2). The spectrotemporal structures of the individual sound pulses cover a broad range for all parameters, as shown in figure 3-21. Over the course of the series the structure would always progress in the same manner. Figure 3-20 shows a typical spectrogram of a squeal series elicited from ACC. There is a gradual progression from long, steep, high-frequency calls to shorter, flatter and lower frequency calls as they fade away. There are only four examples of this call from a spontaneously vocalising animal. These were from one call series which was the result of a guinea pig accidentally banging its head. Figure 3-22 is the spectrogram of this squeal series, with the spectrotemporal analyses shown in figure 3-21. As with the electrically elicited calls, there is a progression from long, steep and high-frequency to short, flat and low-frequency. In contrast, this progression is not gradual and the sound pulse period is irregular.

The rhythmic delivery of squeal series, as seen in 3-20, has never been observed in adult guinea pigs (Berryman, 1976). However, when pups are alone they emit an ‘isolation peep’ which is very similar to an electrically elicited squeal series, both in spectrotemporal structure and sound pulse rhythm. In this sense these squeal series can be viewed as natural, though they would never be produced by an adult (Kyuhou and Gemba, 1998).

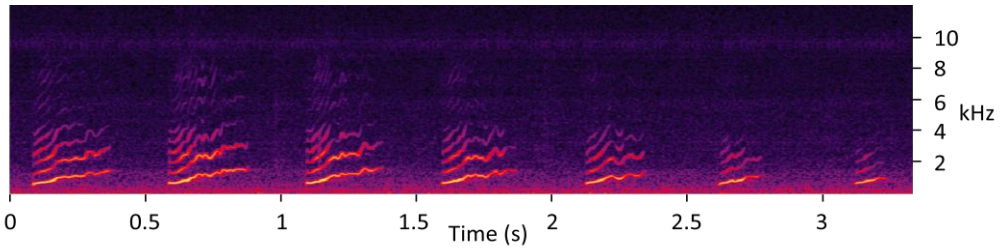



**Figure 3-21** Example spectrogram of a squeal series elicited from ACC.

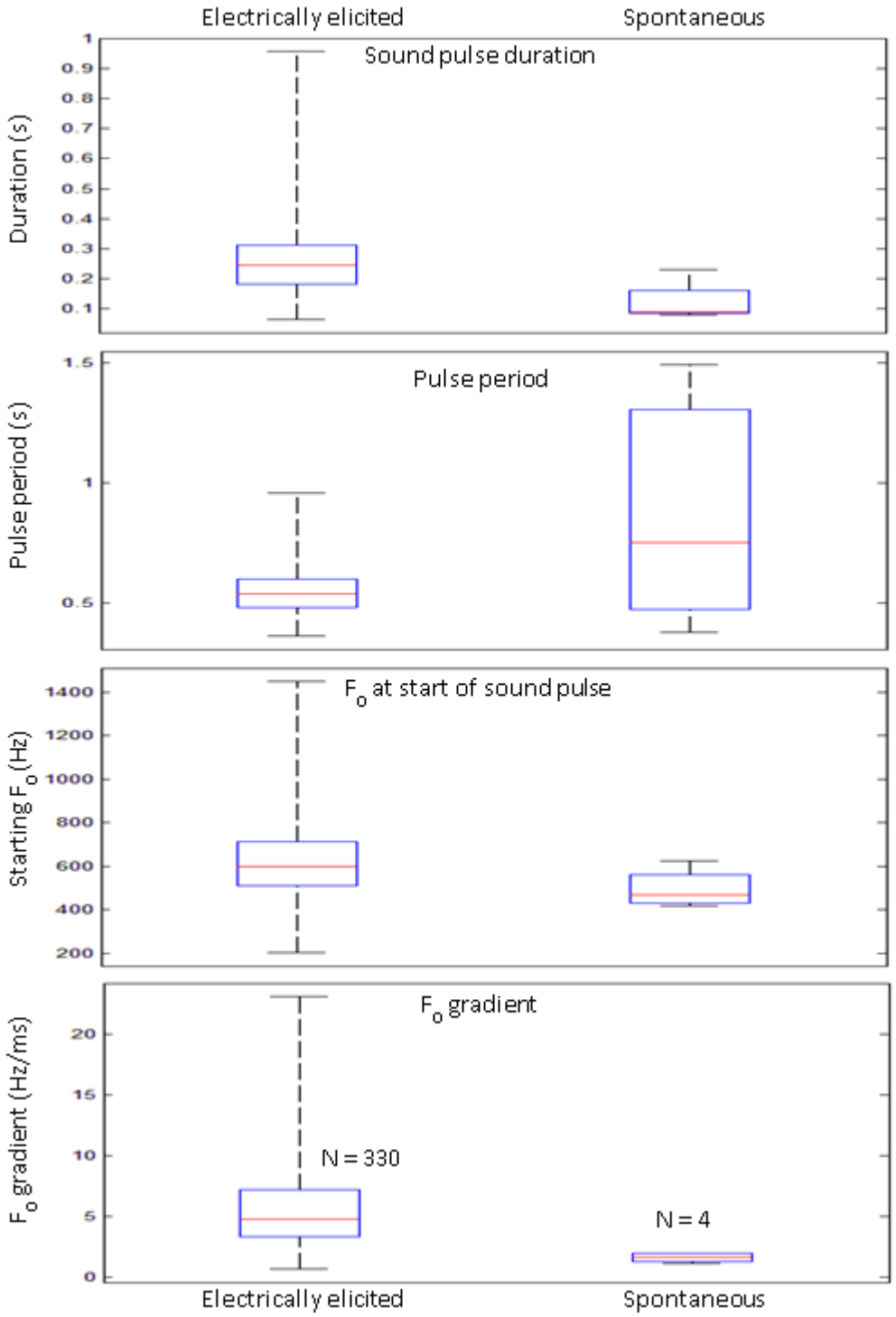



**Figure 3-22** Summarised analyses of electrically elicited and spontaneous squeals. N values indicate the number of individual sound pulses that were analysed.

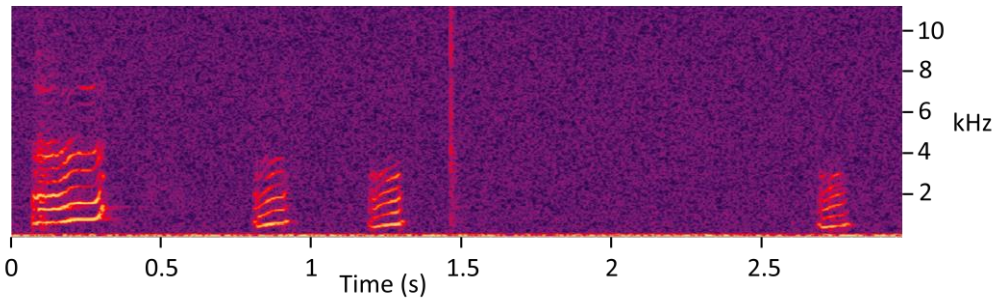



**Figure 3-23** Spectrogram of the sole exemplar of a squeal series from a spontaneously vocalising guinea pig. Made from the original .wav sound file provided by Prof. Grimsley.

### 3.1.11 Scream

Screams are post-stimulus calls that were elicited from the same loci as the squeals, and are a result of higher current stimulation (see section 3.2.2). They could occur individually or as a series of up to five, and were always followed by a squeal series.

Only one scream was recorded by Grimsley. This was followed by the squeal series described above. Screams, though, can be produced in series by spontaneously vocalising animals (Berryman, 1976).

In both the electrically stimulated screams (figure 3-23) and the spontaneously produced scream (figure 3-24) there is a distinctive transition in the gradient, after which follows a flatter, and higher energy portion. This was used to distinguish between screams and squeals. To improve the accuracy of the comparisons, separate analyses were performed on these two sections to each call. These are shown in figures 3-25 and 3-26 for the electrically elicited and spontaneous calls, respectively.

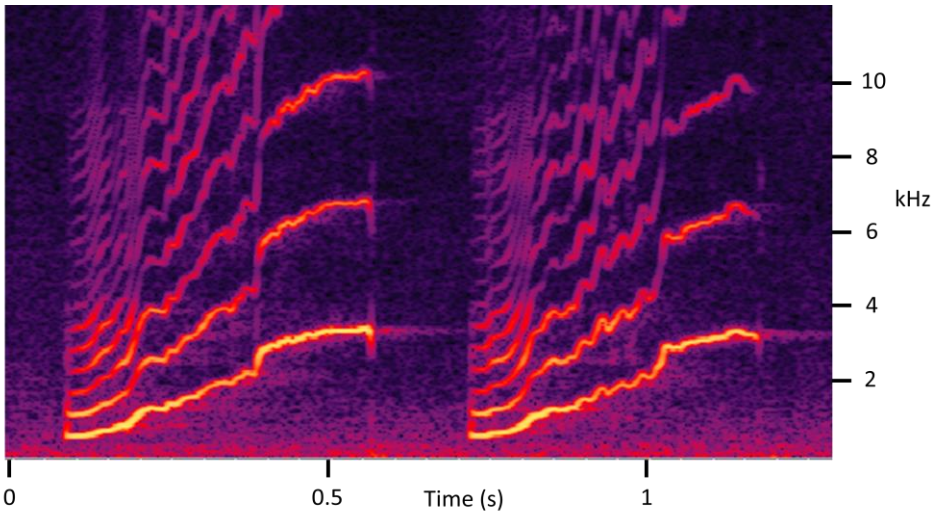



**Figure 3-24** Example spectrogram of screams elicited from ACC.

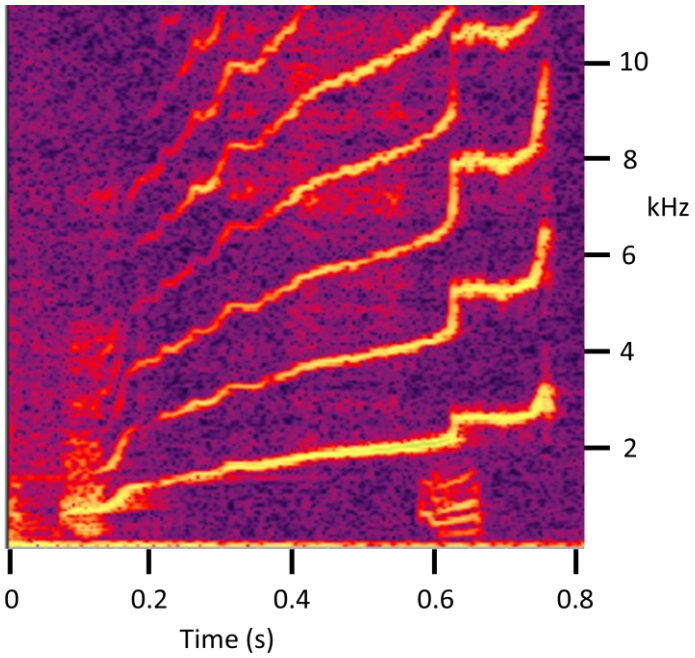



**Figure 3-25** Spectrogram of the sole exemplar of scream from an awake, spontaneously vocalising guinea pig. Spectrogram made from original .wav sound file.

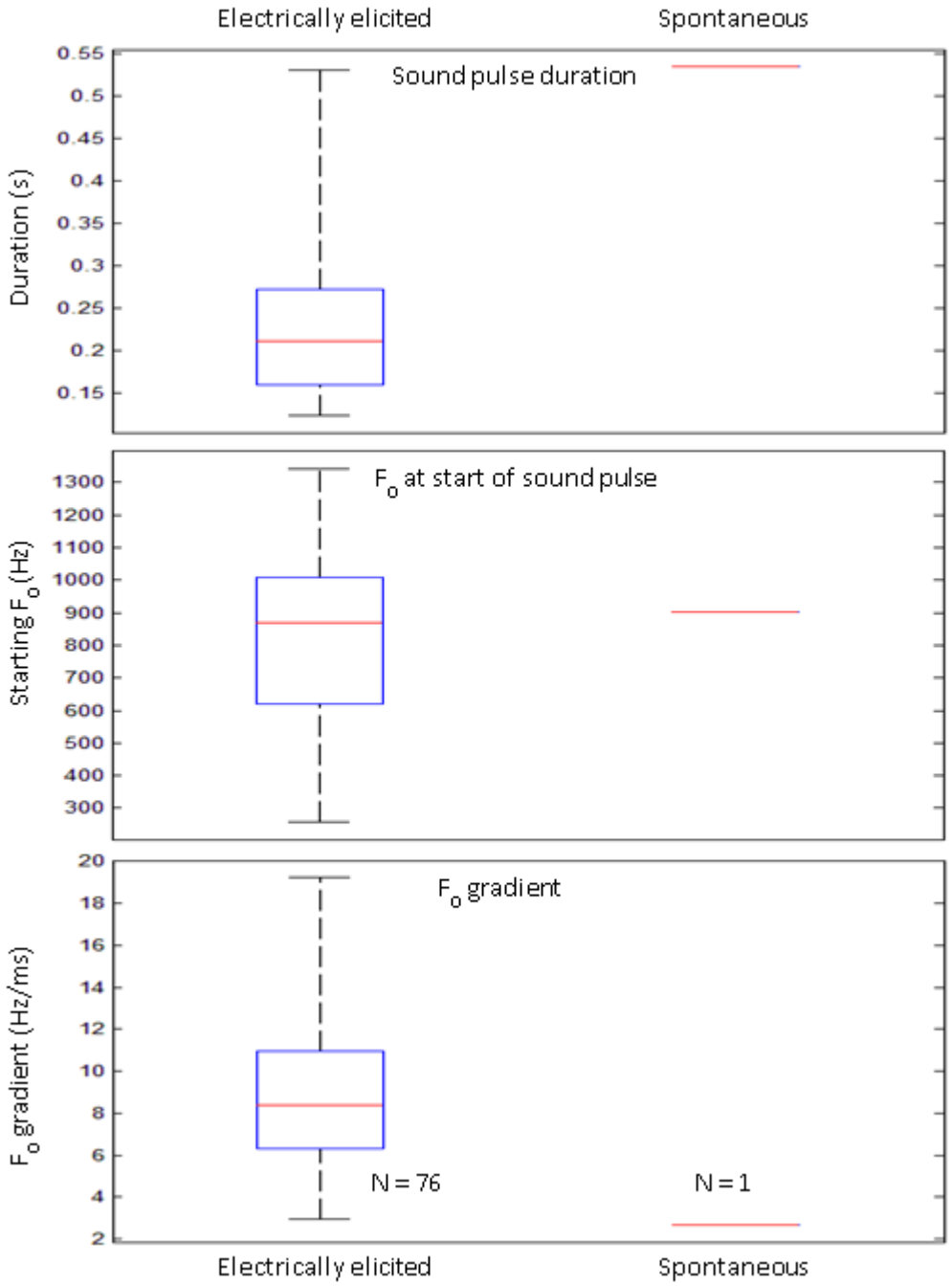



**Figure 3-26** Summarised analyses of the initial portion of the electrically elicited and spontaneous screams. N values indicate the number of individual sound pulses that were analysed.

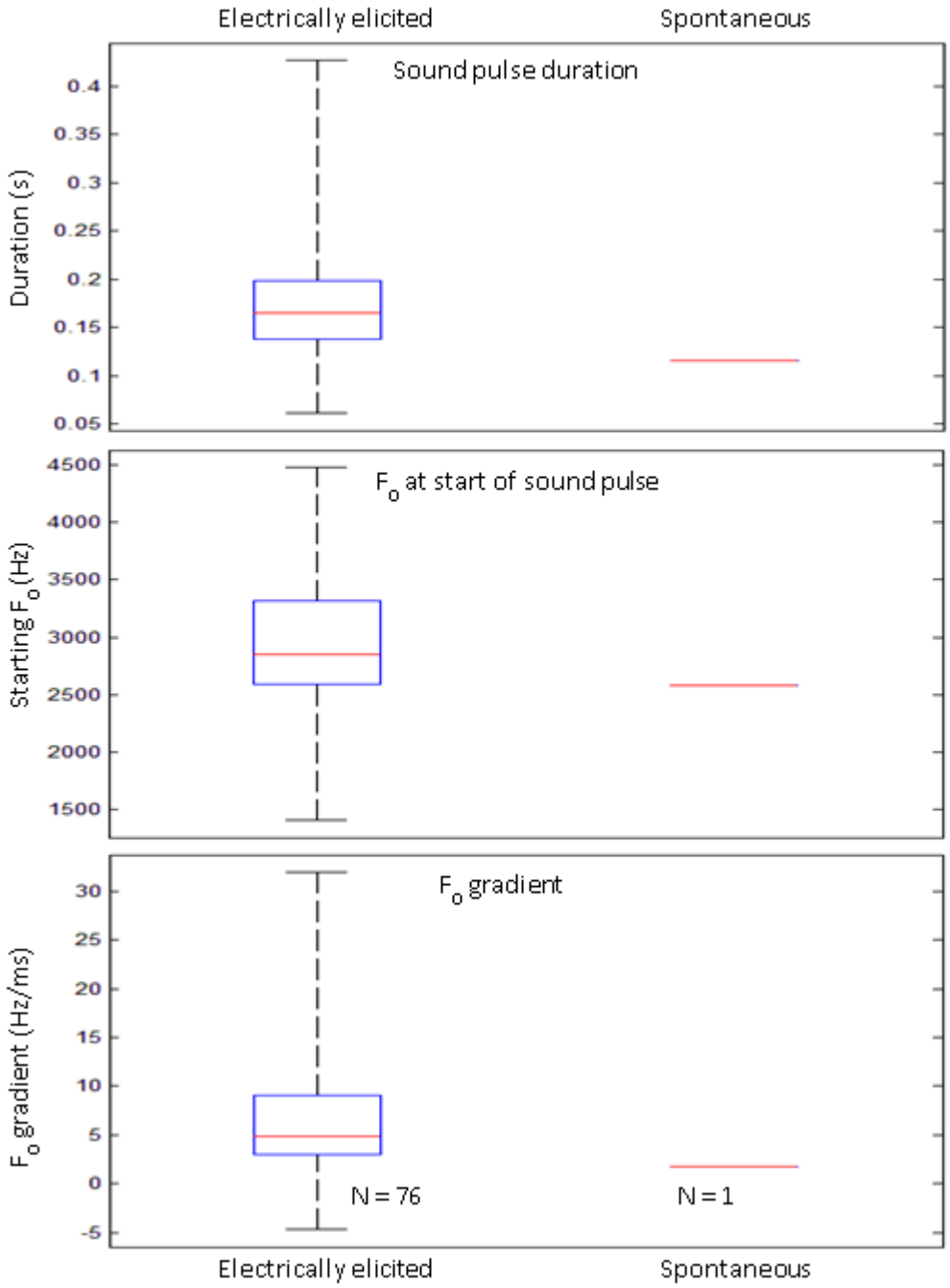



**Figure 3-27** Summarised analyses of the final portion of the electrically elicited and spontaneous screams. N values indicate the number of individual sound pulses that were analysed.

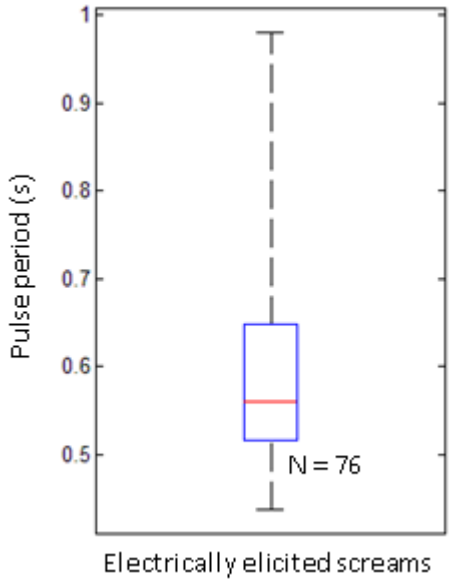



**Figure 3-28** Analyses of the pulse period of the electrically elicited screams, calculated from the undivided screams. N value indicate the number of individual sound pulses that were analysed.

### 3.1.12 Classification of the long duration calls

In contrast to the classification for the short calls, the long duration calls can be classified on the basis of spectrotemporal structure of individual sound pulses. Figure 3-29 shows the summarised analyses of the long duration electrically elicited call types.

Two call types – whine and whistle – have flat gradients yet occupy distinct $F_0$ ranges: all whine exemplars are below 2 kHz; all the whistles are above this $F_0$. The remaining two call types – scream and squeal – both have positive $F_0$ gradients. The squeal is similar, in all parameters, to the initial portion of the scream. They are discriminated on the basis of the high frequency, flatter gradient, final portion of the scream.

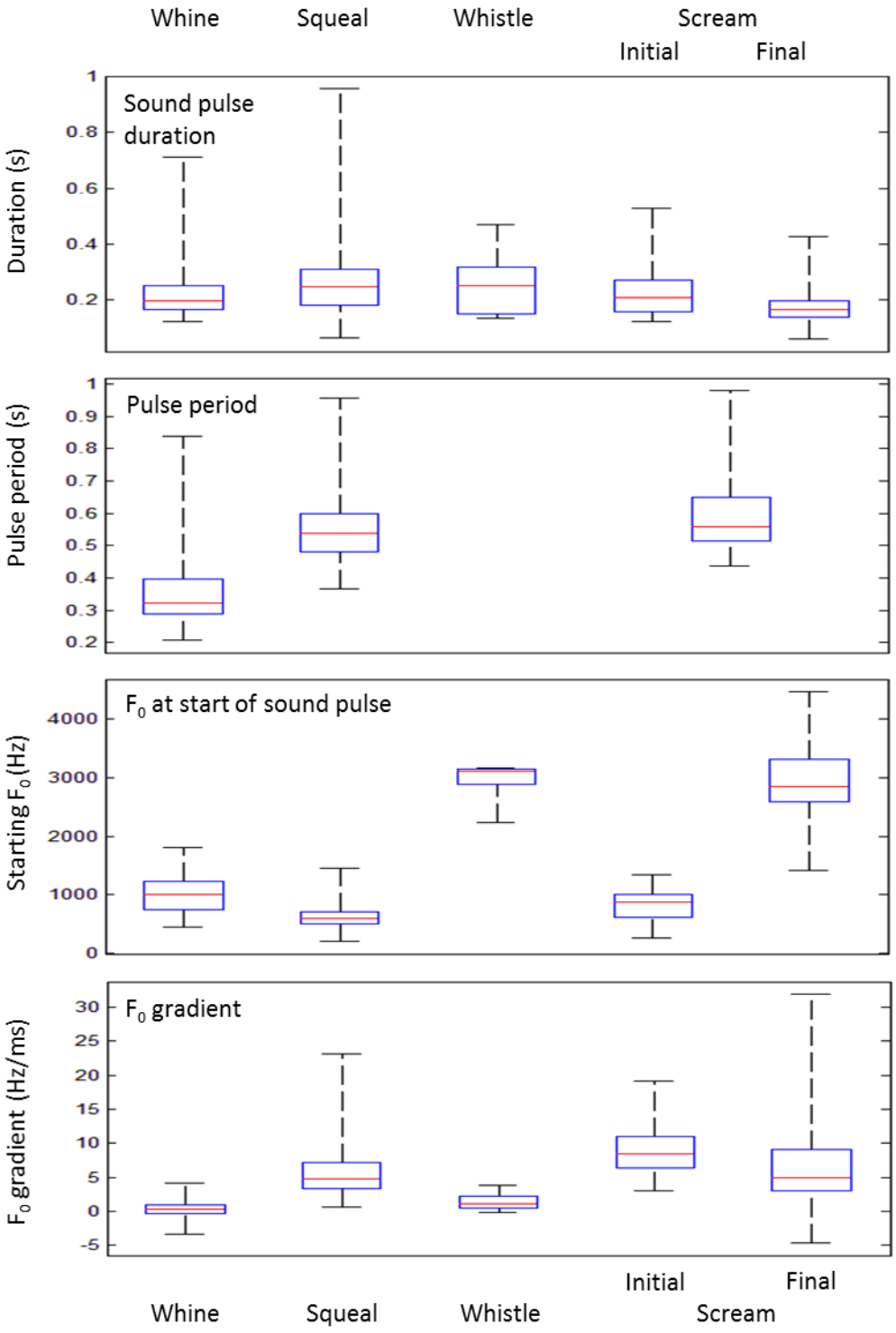



**Figure 3-29** Collated exemplars from all brain areas of the 4 classes of long duration call.

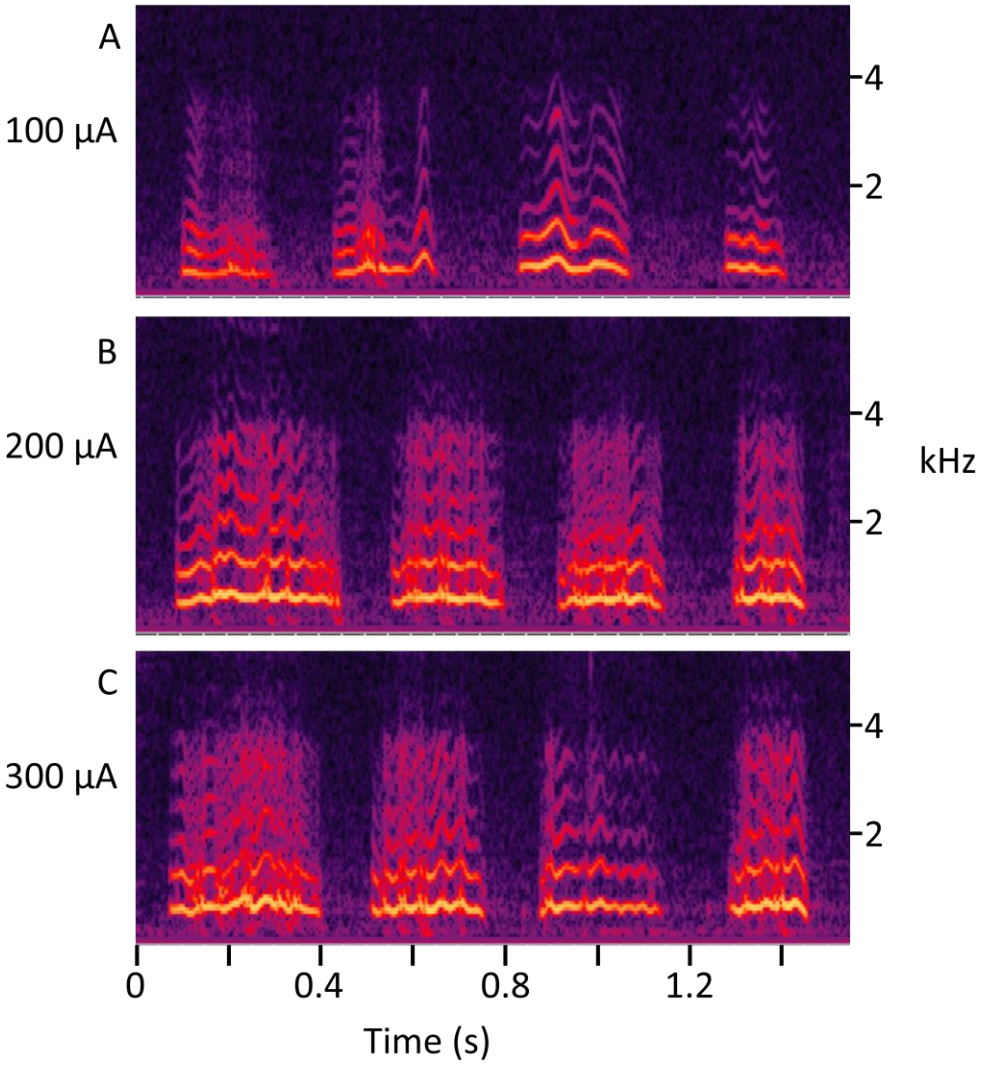
### 3.2 Stimulation parameters

Only one group has elicited vocalisations from anaesthetised guinea pigs (Kyuhou & Gemba, 1998, 1999). For this reason, the current experiments followed their protocol. Changes to stimulation current affected three of the calls, whereas changes in stimulation frequency had no effect.

When using electrical stimulation it is not possible to say whether the neural activation is of cell bodies or fibres of passage. Others have used chemical stimulation, such as glutamatergic or cholinergic agonists, to elicit vocalisations. In addition to activating cell bodies exclusively, these techniques also provide information as to the specific neuron type responsible for vocal production (Brudzynski and Bihari, 1990; Jürgens and Richter, 1986; Lu and Jürgens, 1993; Manteuffel et al., 2007). Increasing the stimulation current leads to an increased area of activated neurons (Rowley et al., 1996). Three calls – scream, squeal and whine – could be modified by stimulation current. These are discussed in detail below.

#### 3.2.1 Modification of whines by stimulus current

The spectrotemporal structure of the whine vocalisation could be altered by changing stimulation current. Higher currents gave longer duration sound pulses with higher F0. Figure 3-32 A-C shows spectrograms of current change in one locus of the amygdala and the corresponding analysis is shown in figure 3-32 D.

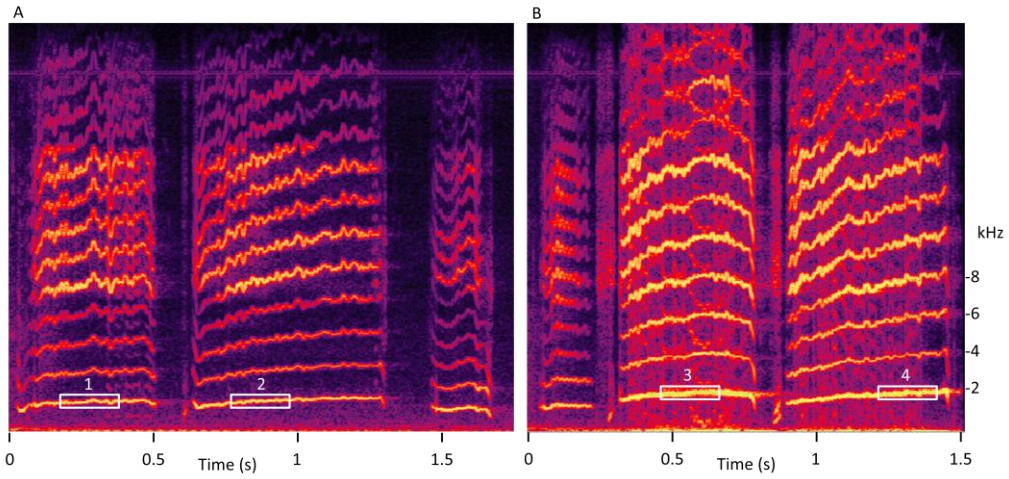



D

| | Mean Duration (s) | Mean F0 (Hz) |
|---|---|---|
| 100uA | 0.17 | 463 |
| 200uA | 0.23 | 604 |
| 300uA | 0.24 | 642 |

**Figure 3-30** A-C: Example spectrograms of an amygdalar whine response to stimulation current. D: summarised analysis from A-C.

Figure 3-33 shows the same amygdala locus stimulated at 300 µA (A) and 400 µA (B). It illustrates how the intensity of sound can be increased by stimulation current (data shown in 3-33C).




| C | | Duration (s) | F0 (Hz) | Gradient (Hz/ms) | Window RMS (V) |
|---|---|---|---|---|---|
| Sound pulse: | 1 | 0.47 | 1387 | 0.60 | 0.04 |
| | 2 | 0.65 | 1412 | 0.68 | 0.04 |
| | 3 | 0.45 | 1834 | 0.56 | 0.42 |
| | 4 | 0.54 | 1598 | 1.03 | 0.27 |

**Figure 3-31** Stimulation current increases sound intensity. Spectrograms of whines elicited from the same location at 300 µA (A) and 400 µA (B), with the corresponding data in the table (C).

### 3.2.2 Modification of scream/squeal series by stimulus current

Post-stimulus screams and squeals were always produced from the same anatomical loci. At high current between 1 and 5 screams were produced followed by squeals which gradually decreased in duration, $F_0$ and gradient. Lower current stimulation would yield only squeals. This effect is illustrated in figure 3-34.

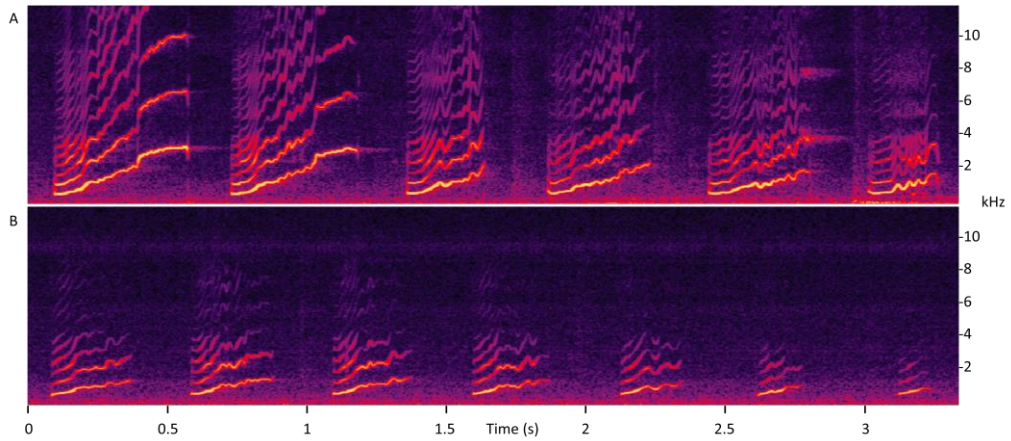



**Figure 3-32** Spectrograms of scream/squeal series elicited from the same locus in ACC. A) Stimulated at 200 µA giving two screams followed by squeals. B) Stimulated at 100 µA giving only squeals.

### 3.2.3 The effect of anaesthesia

The ability to elicit a vocalisation was highly dependent on the level of anaesthesia. On several occasions, an electrode in a proven vocal production locus would cease to give a vocalisation following the administration of a bolus of anaesthetic. Its ability to give a vocalisation would return over the course of 30-60 minutes; requiring higher current to elicit vocalisations at deeper levels of anaesthesia. Due to the confounding effect of the anaesthetic, it was not possible to use the threshold electrical current for eliciting a vocalisation as a way of mapping proximity to vocal production centres.

It is possible that certain call types are more susceptible to anaesthesia that others. This may account for the incomplete repertoire. Further study using chronically implanted electrodes in awake animals would illuminate this issue.

Given that, with the exception of chutter, all the electrically evoked vocalisations are very similar to natural calls it is unlikely that the anaesthesia had any effect on the spectrotemporal structure of the vocalisations.

### 3.2.4 The effects of repeated stimulation

The during-stimulation calls elicited from the amygdala, the hypothalamus and the anterior cingulate cortex habituated to repeat stimulations. That is, after several pulse train repetitions, stimulation of the brain locus would fail to elicit a call. The ability to produce a call would return after approximately 2-5 minutes. This suggests that the habituation is transient and not caused by cell damage. Unlike the above areas, repeated stimulation of the PAG did not lead to habituation. Conversely, the post-stimulus scream/squeal series were enhanced by repeat stimulations. This was true of all loci from which this call series was elicited. The other post-stimulus call, toothchatter, showed high sensitivity to habituation. In all but one locus, this call could only be elicited once.

The biological basis for these effects cannot be inferred using microstimulation alone. Alternative experimental approaches that could provide answers are discussed in section 7.

### 3.2.5 Frequency of the stimulation pulse train

I initially postulated that the sound pulse rate of flat chutter was phase locked to the frequency of the electrical stimulation. Variation of stimulation frequency, however, did not alter the pulse rate of these calls. It was later discovered that this finding agrees with the published literature (Jürgens, 2002). For this reason extensive analysis was not performed. Figure 3-35 shows data for PAG-elicited flat chutter. In agreement with the published literature, changing the stimulation frequency had no effect on the sound pulse frequency of the call. In both animals, the stimulation

frequency was doubled and the resulting vocalisation remained within a very narrow (4 Hz) range of variation.

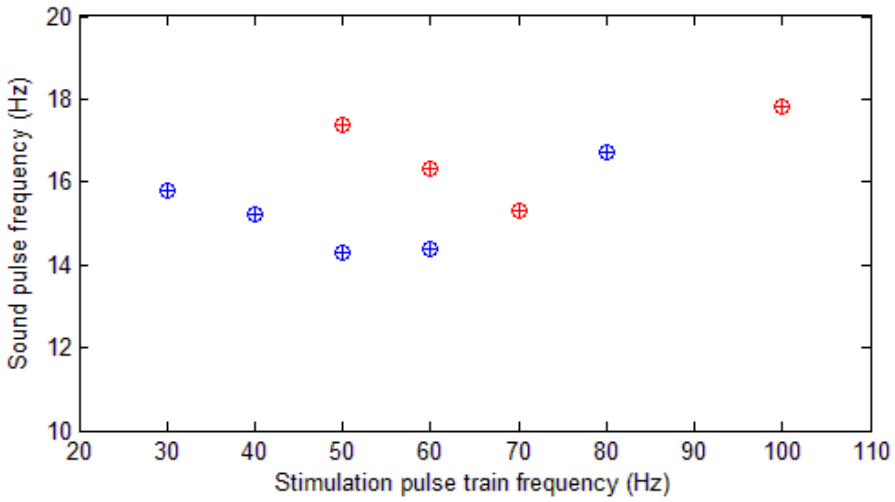



**Figure 3-33** The sound pulse rate of flat chutter is unaffected by the frequency of the microstimulation pulse train. Calls elicited from the PAG in two animals (red and blue marks).

### 3.3 Brain structures that yield vocalisations

Figure 3-36 summarises the brain areas from which the calls could be elicited. In addition to these four areas, scream/squeal series could be elicited from a range of other brain regions (see section 3.3.5).

| | Brain area: | | | |
|---|---|---|---|---|
| Call type: | ACC (12) | Hypothalamus (19) | Amygdala (42) | PAG (8) |
| Toothchatter | | 3 | 5 | |
| Whistle | | | | 1 |
| Whine | | 1 | 40 | 6 |
| Chut | 4 | 6 | | 4 |
| Rising chutter | | | | 3 |
| Flat chutter | | 1 | | 1 |
| Scream | 12 | 19 | 11 | 7 |
| Squeal | 12 | 19 | 11 | 7 |

**Figure 3-34** Summary table showing the number of animals from which each call could be elicited in each brain area. The total number of attempts to evoke calls from a given brain area are shown in parentheses.

Figure 3-37 shows the locations, as determined by histology, of all the call production loci, except for scream and squeal. As previously mentioned, loci from which screams and squeals were elicited are one and the same. Owing to their numerous and widespread nature, a separate figure (3-38) was necessary. The guinea pig atlas did not detail the division of cingulate cortex (Rapisarda and Bacchelli, 1977). The labels of ACC were added after comparison with a rat brain atlas which described the whole brain, including cortical structures (Paxinos and Watson, 1998).

### 3.3.1 PAG elicited vocalisations

According to current understanding, the PAG of every mammalian species ought to contain loci from which the full vocal repertoire can be elicited (Gruber-Dujardin, 2010). Of the eight vocalisations produced in the present study, all except TC could elicited from the PAG. TC loci were the rarest of all the vocalisations and the PAG was

the least extensively probed nuclei. It remains possible, therefore, that TC loci exist in the guinea pig PAG. A more thorough investigation would be required to find them.

Two vocalisations – whistle and rising chutter – were elicited only from the PAG. The current understanding of vocal production suggests that there would be loci in other brain areas that would yield these calls. Again, more thorough and extensive study would be required to confirm this prediction.

Previous researchers have shown that the PAG is compartmentalised. Again, the data presented here agree with this. On several occasions, an electrode array at a given location could yield different call types on its anterior and posterior electrode pairs. Furthermore, advancing an electrode array could yield up to three different call types on the same electrode pair within 600 µm. One such example can be seen in figure 3-37B. Here, the same electrode pair moved from whine to rising chutter to flat chutter with each 200 µm step.

In contrast to all the other nuclei in this study, scream/squeal loci in the PAG are scant. Though there is no apparent explanation for this difference.

### 3.3.2 Hypothalamic vocalisations

Six vocalisation types were elicited from the hypothalamus. This variety is unsurprising given that this nucleus receives input from vocal production regions in amygdala and ACC.

This study did not accumulate enough data for the during-stimulus calls or TC to state whether these loci were confined to particular subnuclei. Martin (1976) – who had an 'unclassified' category for call types in addition to the three specific types that he

classified by ear – found that the call loci were distributed throughout the hypothalamus. The same has been shown in squirrel monkey hypothalamus (Jürgens, 1998).

The post-stimulus scream/squeal series could be elicited from large portions of the hypothalamus as well as surrounding areas. In the same way, there did not appear to be any bias towards particular subnuclei.

### 3.3.3 Amygdalar vocalisations

Here, for the first time, vocalisations have been elicited from the guinea pig amygdala. Furthermore, this is the first report of more than one amygdalar call type in a nonprimate species. Evidence for two separate amygdala vocalisation pathways was provided in the same study using squirrel monkey. As previously discussed (see section 1.7) each of these pathways had been shown separately by different researchers in different rodent species.

In squirrel monkey, calls associated with self-confidence and aggression were shown to follow the indirect descending pathway via the stria terminalis, whereas calls associated with fear and aversion were shown to follow the direct amygdalofugal pathway (Jürgens, 1982). The two amygdalar calls in the present study are indicators of different motivational states: TC is associated with self-confidence and aggression; whine with fear and aversion (Berryman, 1976). It might be hypothesised that the descending pathways of GP vocalisations are similarly divided based on the emotional meaning of the call type. That is, TC following the indirect pathway and whine following the direct pathway.

With reference to the audio-vocal part of this thesis: In addition to its potential connectivity with auditory cortex, the basal amygdala in this study only produced whines. This adds some confidence that the only the whine production circuitry will be active. Although, due to the nature of TC loci, this is not conclusive.

### 3.3.4 ACC vocalisations

All the ACC call loci in the present study were in the ventral portion. Squeals have been elicited from guinea pig ACC before, and with similar distribution to those shown here (Kyuhou and Gemba, 1999). In squirrel monkey, however, call production loci are distributed over the whole of ACC. The reasons for this disparity are unclear.

### 3.3.5 Vocalisations from other brain areas

Scream/squeal loci were also found in a number of additional brain areas. These included various thalamic nuclei, the anterior portion of the posterior cingulate cortex, the lateral septum and portions of midbrain surrounding PAG. The extensive distribution of scream/squeal loci is shown in figure 3-38. Their diffuse nature prevents any definite conclusions from being drawn.

### 3.3.6 Global distribution of call types

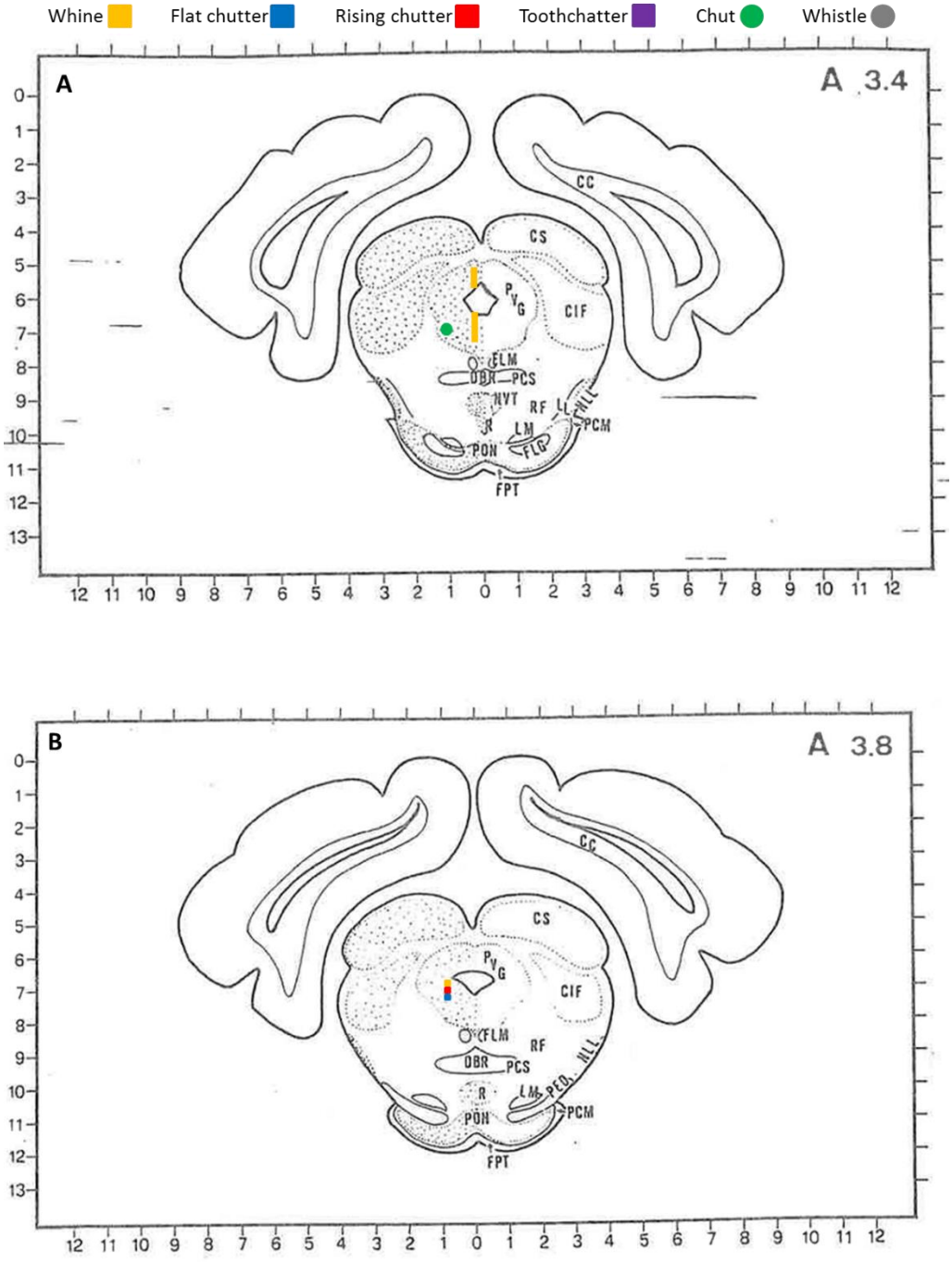
Figure 3-37 shows that, whilst the amygdalar vocal production loci are exclusively aversive, those in ACC are exclusively non-aversive. In contrast, PAG and hypothalamus contain a mixture of aversive and non-aversive call types. In squirrel monkey, however, all these brain areas yield a variety of aversive and non-aversive

call types (Jürgens, 1998). To date, this is the only research with which the results of the present study can be compared. The ACC/amygdala dichotomy shown here may be a true feature of the vocal production systems in GPs and other nonprimates. It is also possible, however, that these results are the coincidental effects of anaesthesia and stimulation parameters. These are discussed further in section 7.1.5.

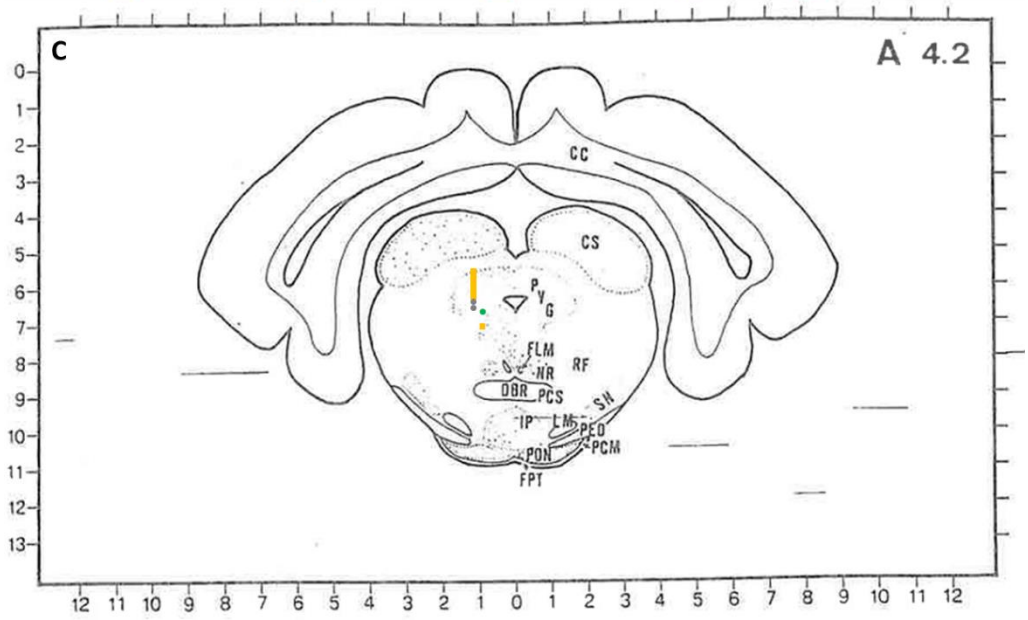
Abbreviation list for figures 3-35 and 3-36:

| | | | |
|---|---|---|---|
| AB | Basal amygdala | LS | Lateral septum |
| AC | Anterior commissure | LT | Lateral thalamus |
| ACC | Anterior cingulate cortex | MD | Mediodorsal thalamus |
| ACE | Central amygdala | LM | Medial lemniscus |
| ACO | Cortical amygdala | MFB | Medial forebrain bundle |
| AD | Anteriodorsal thalamus | ML | Lateral mammillary nucleus |
| AHA | Anterior hypothalamus | MM | Medial mammillary nucleus |
| AL | Lateral amygdala | MT | Mammillothalamic fasciculus |
| AM | Anteriomedial thalamus | NPT | Posterior thalamus |
| AME | Medial amygdala | OC | Optic chiasm |
| AV | Anterioventral thalamus | OT | Optic tract |
| CC | Corpus callosum | P | Paraventricular thalamus |
| CH | Commissure of fornix | PCC | Posterior cingulate cortex |
| CI | Internal capsule | PH | Posterior hypothalamus |
| CIF | Inferior colliculus | PMD | Dorsal praemamillaris |
| CPU | Caudate putamen | PT | Parataenialis nucleus |
| CS | Superior colliculus | PV | Paraventricular nucleus |
| F | Fornix | PVG | Periaqueductal grey |
| FIM | Fimbria of hippocampus | RH | Rhomboid nucleus |
| GL | Lateral geniculate body | RT | Reticular thalamus |
| GM | Medial geniculate body | SC | Suprachiasmatic nucleus |
| GP | Globus pallidus | SM | Stria medullaris of thalamus |
| HPC | Hippocampus | SN | Substancia nigra |
| LH | Lateral hypothalamus | VT | Ventral thalamus |

**Figure 3-35** The locations, as determined by histology, of all the call production loci, except for scream and squeal. Aversive calls are represented with rectangles, non-aversive and neutral calls are represented with circles.



Whine Flat chutter Rising chutter Toothchatter Chut Whistle



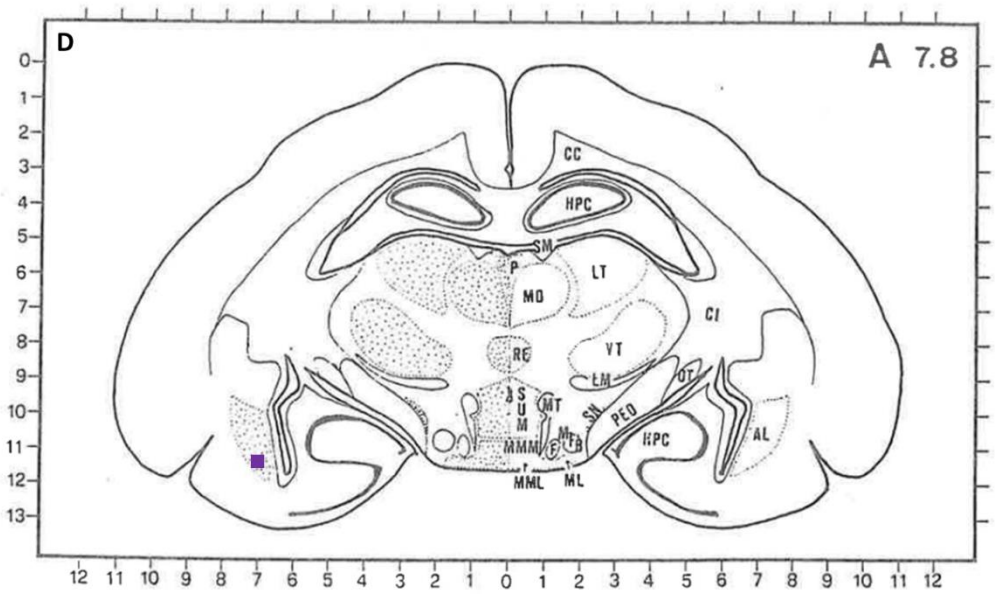
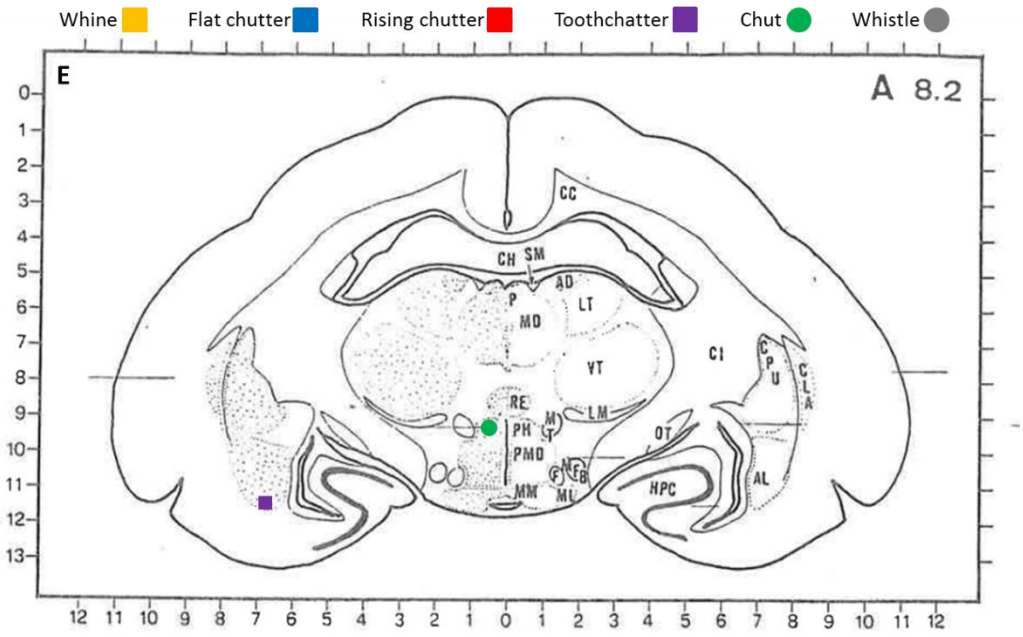




Plate XII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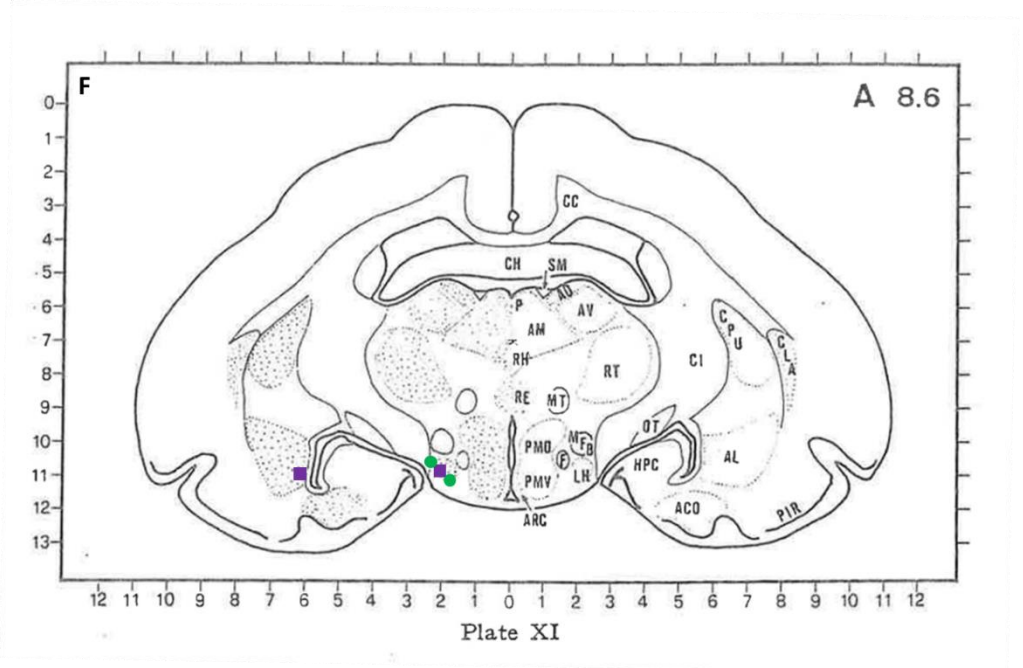
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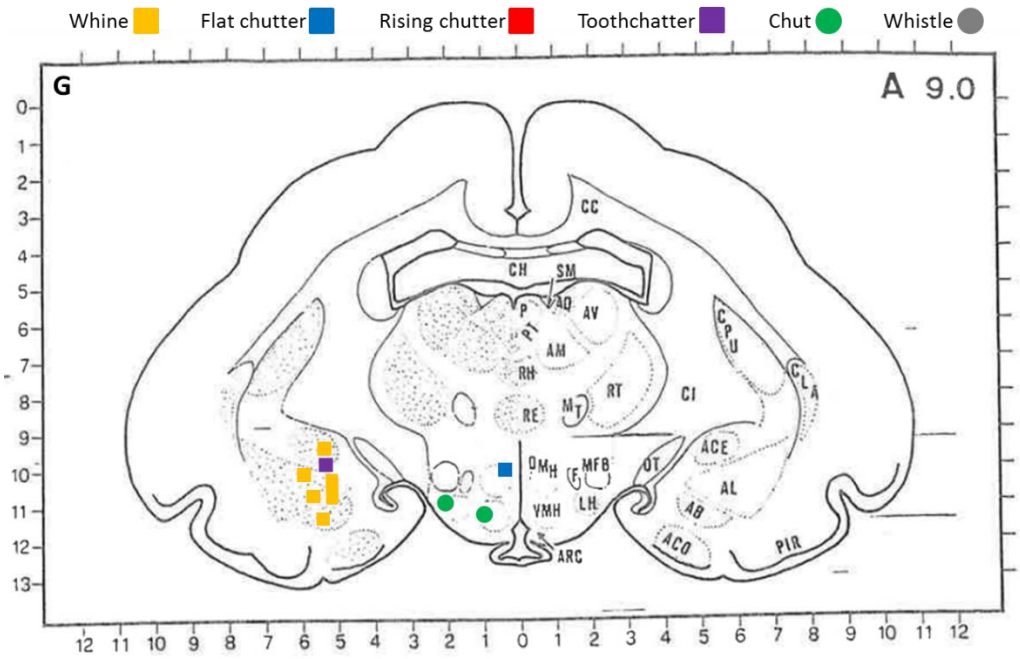
Plate XI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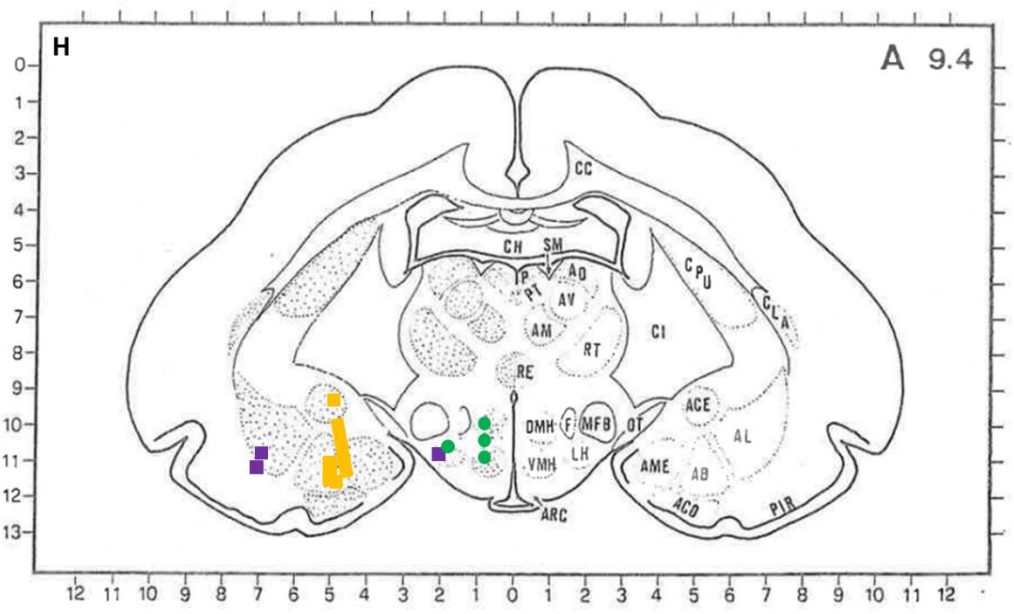
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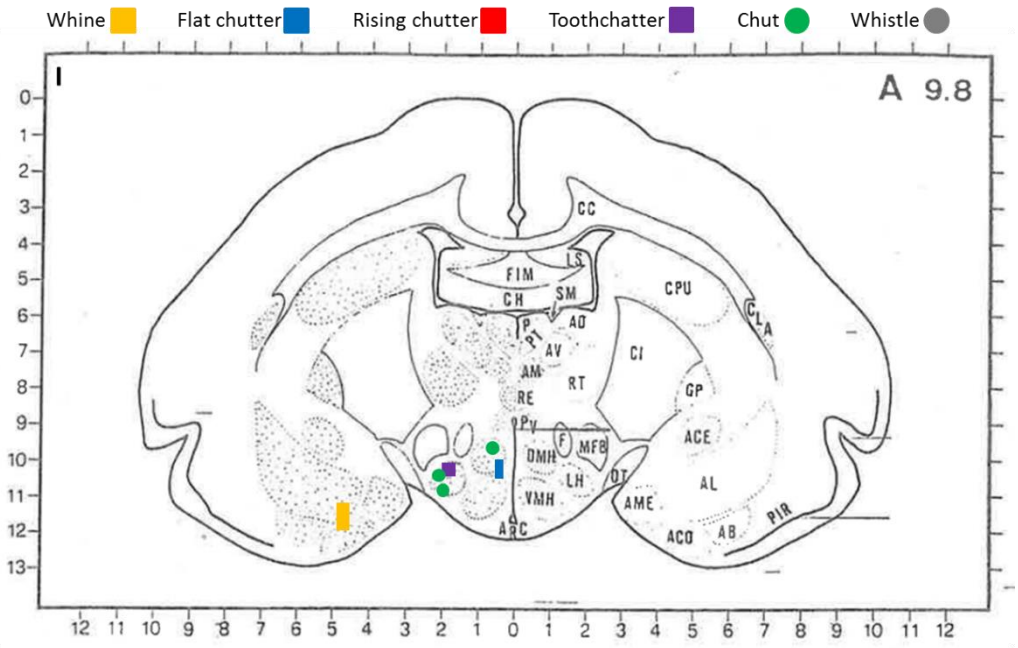
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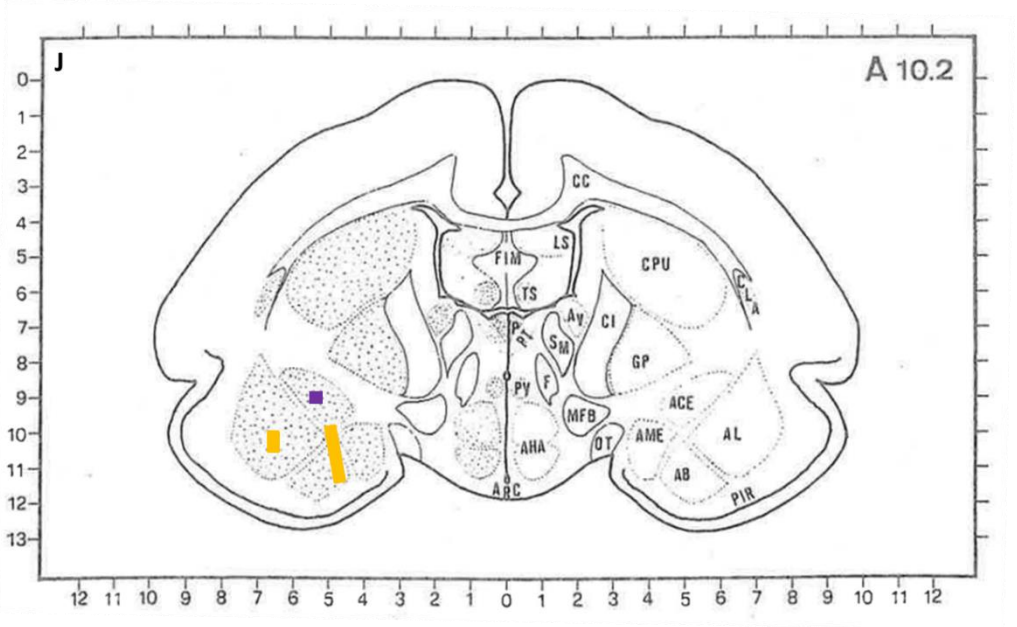
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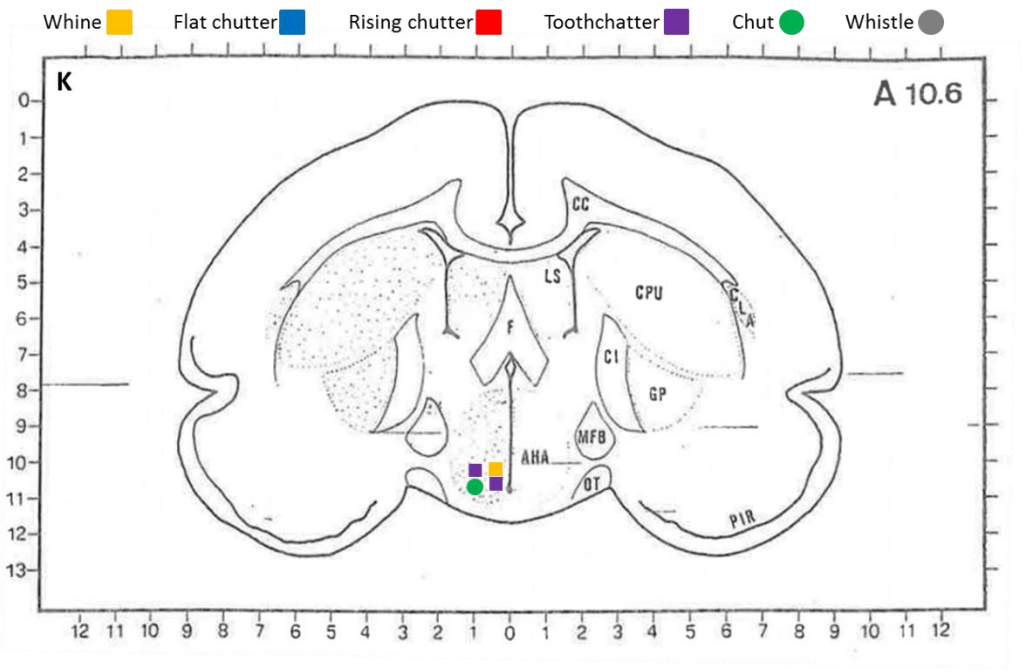
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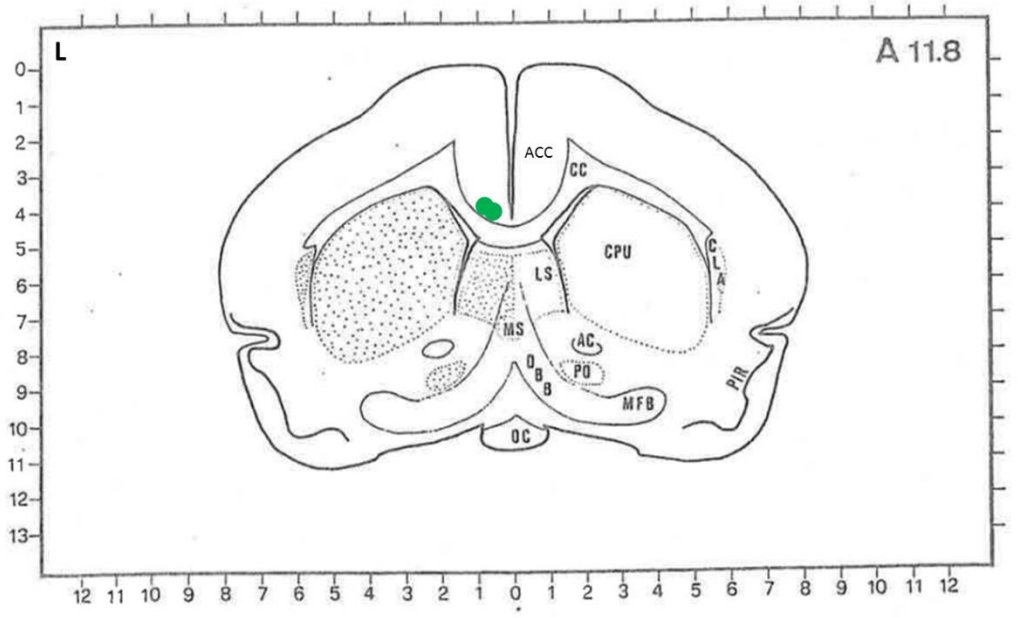
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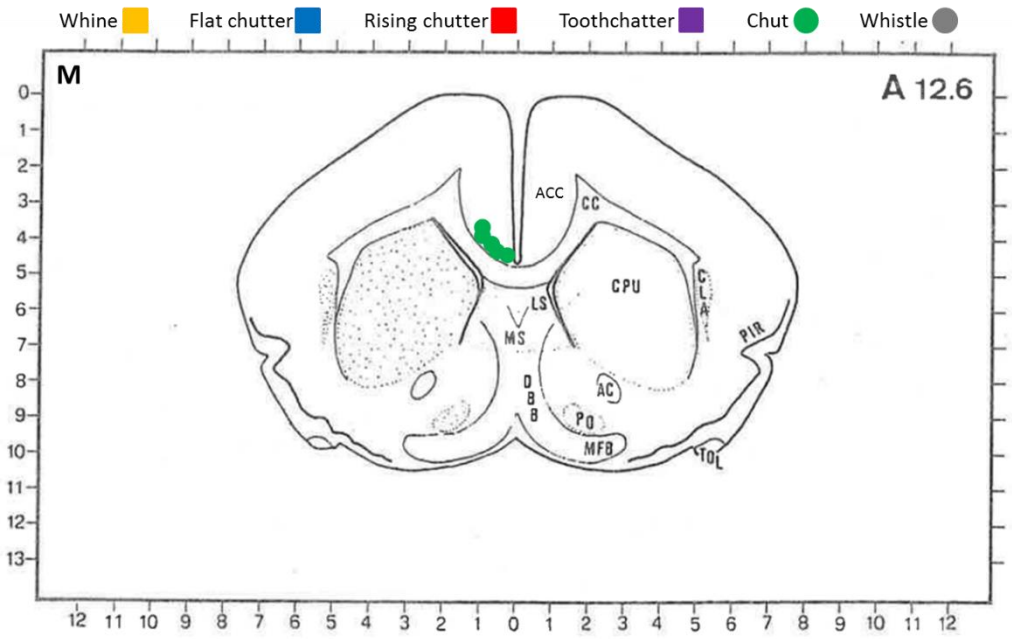
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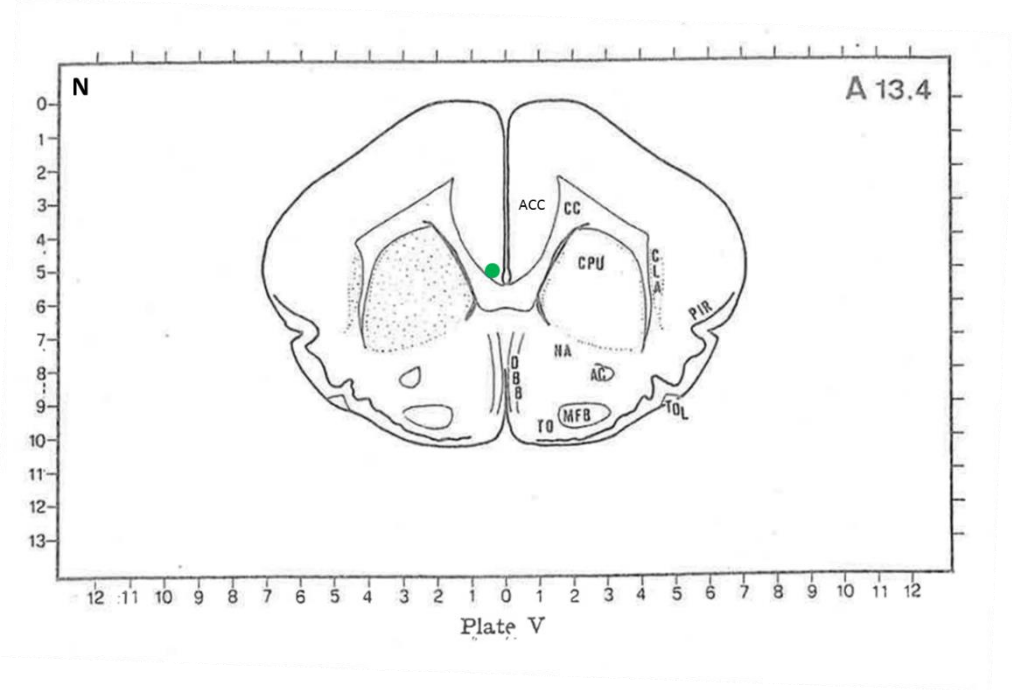
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Plate V

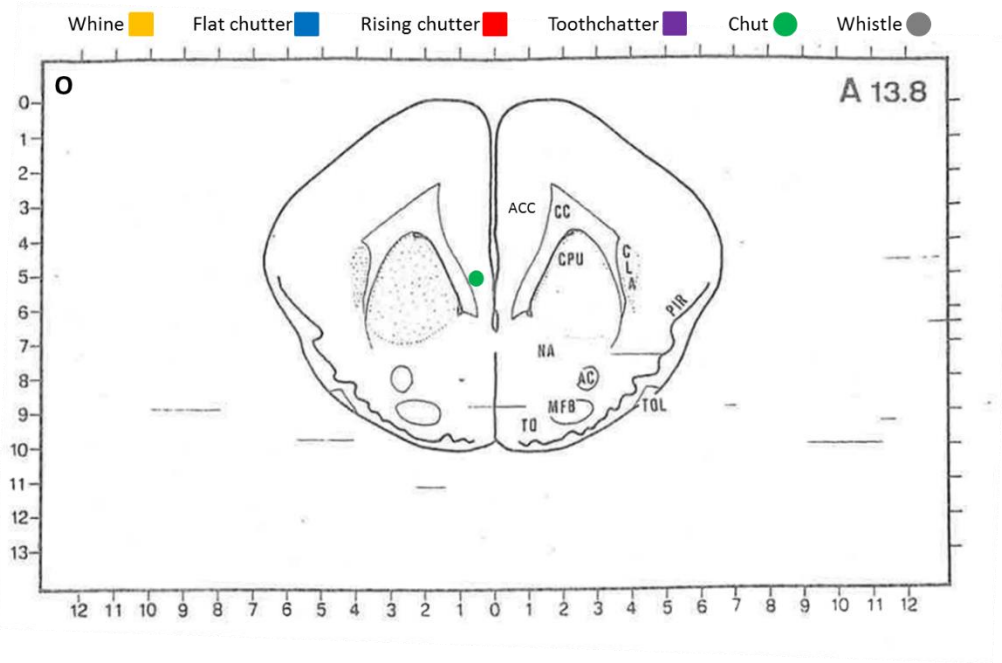
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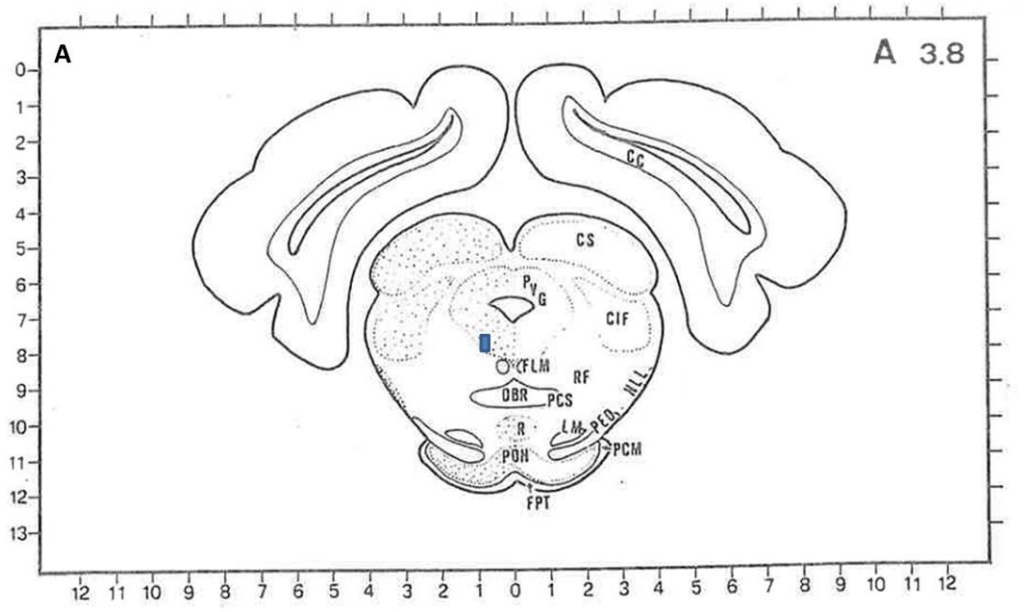
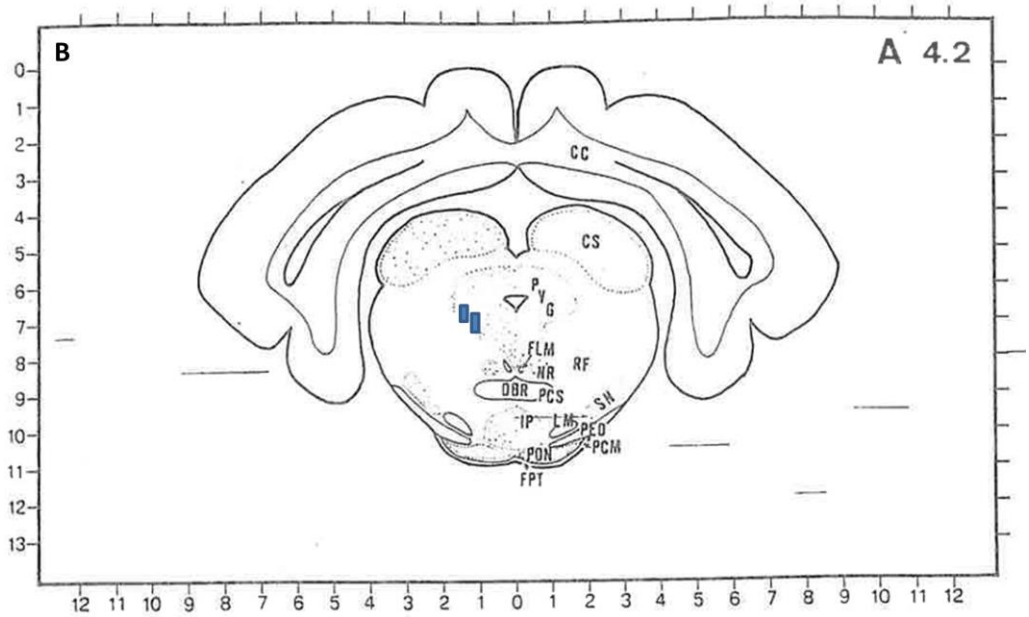
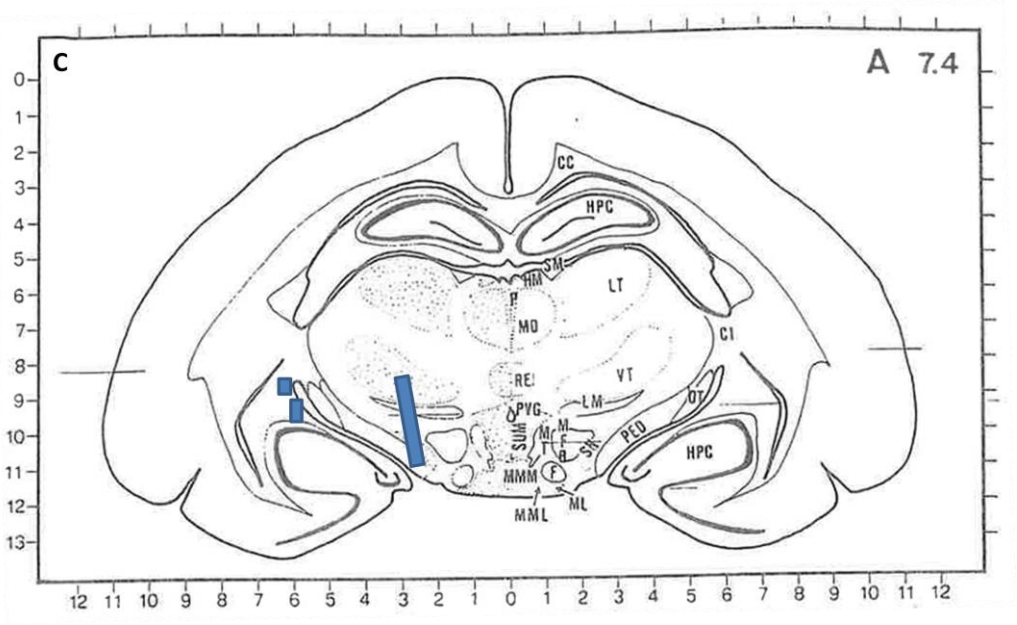
**Figure 3-36** The call production loci, as determined by histology, of scream/squeal call.

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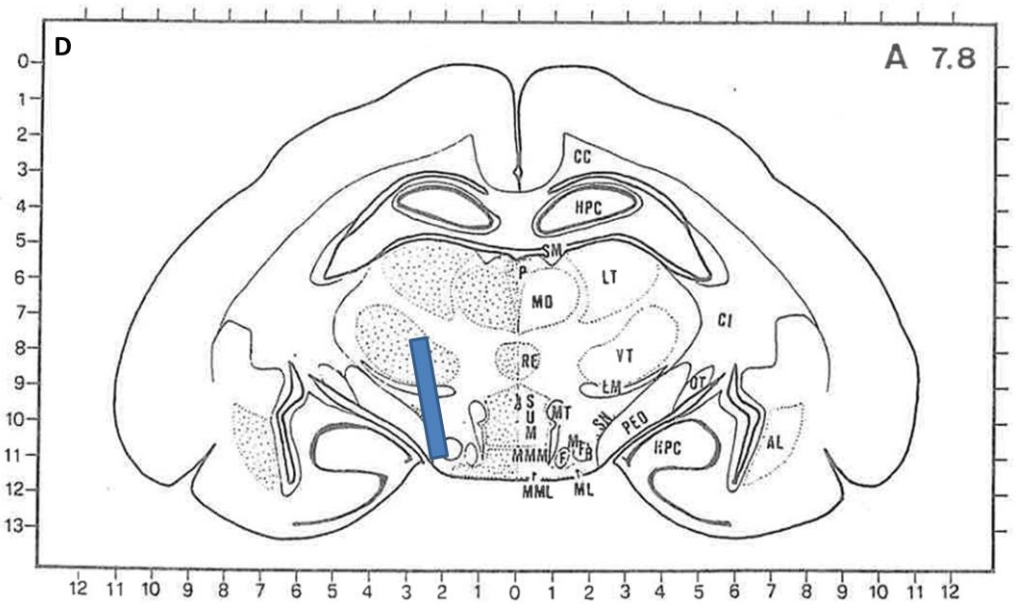
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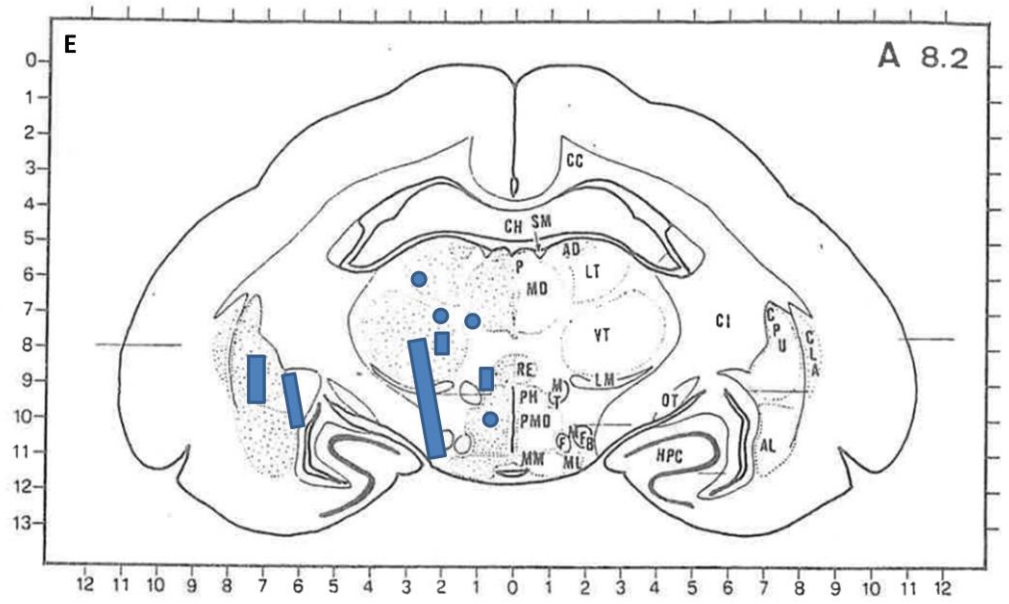
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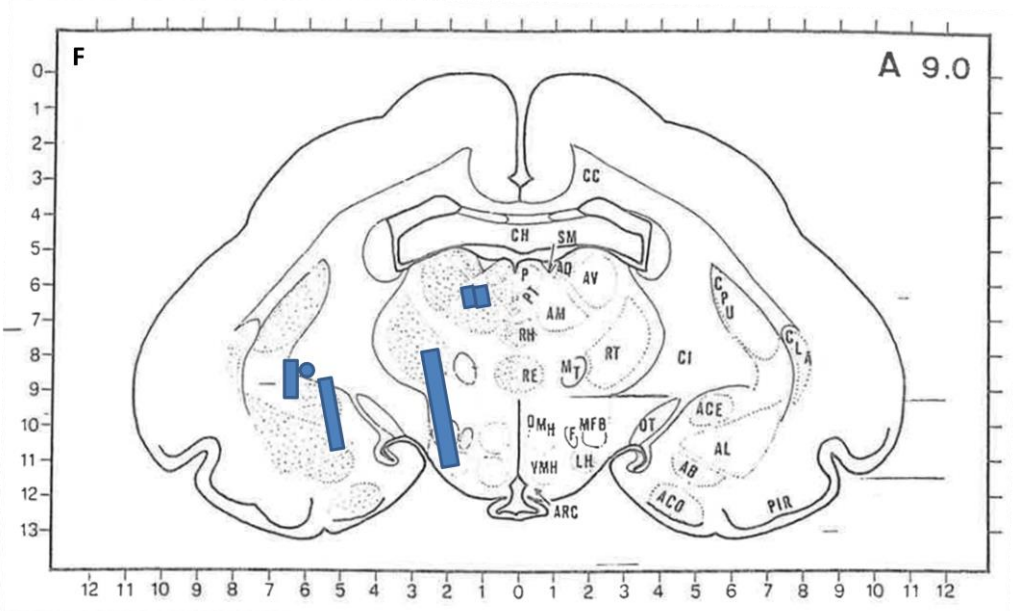
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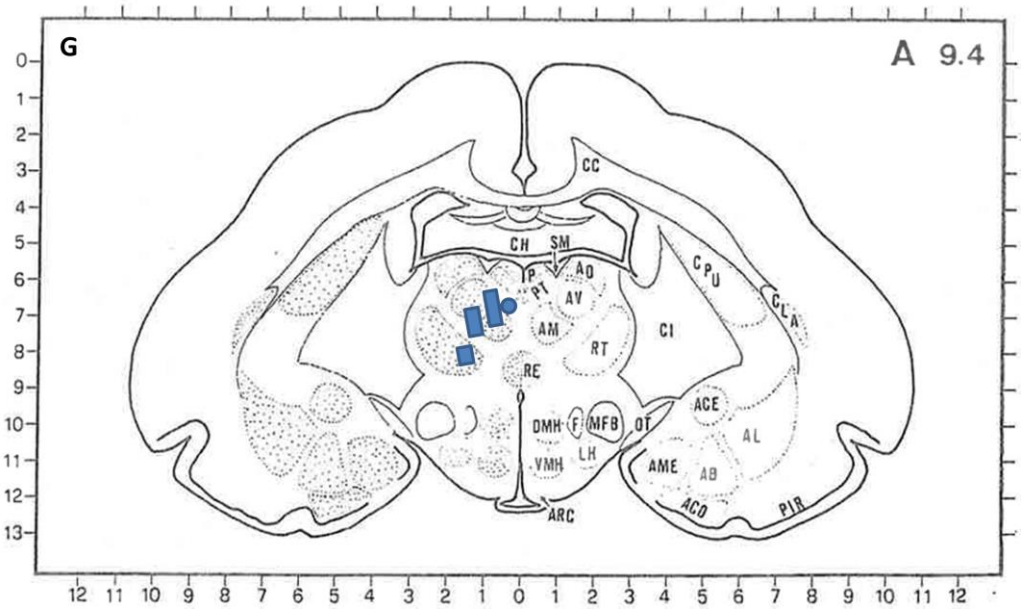
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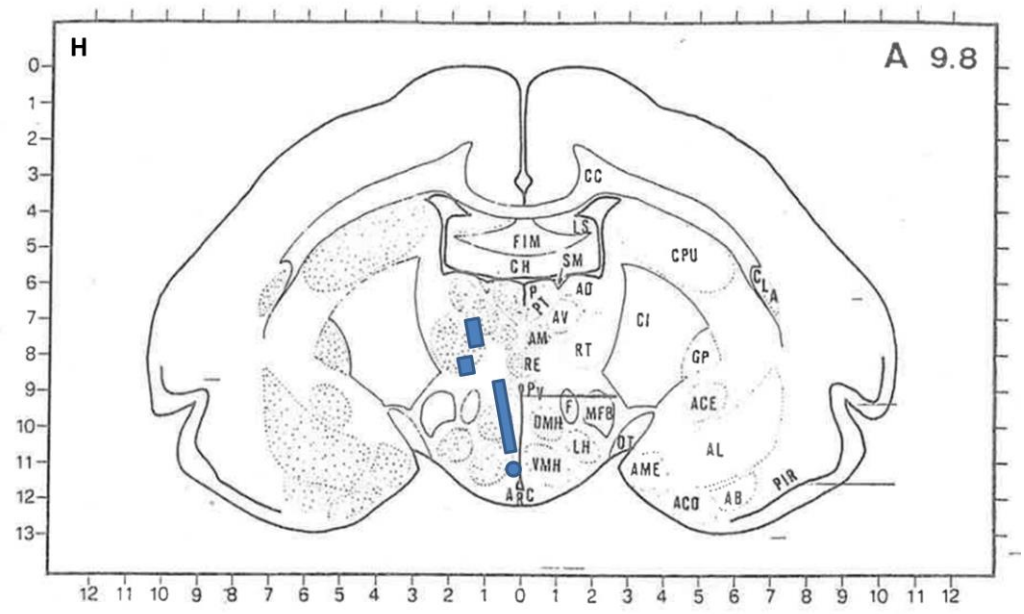
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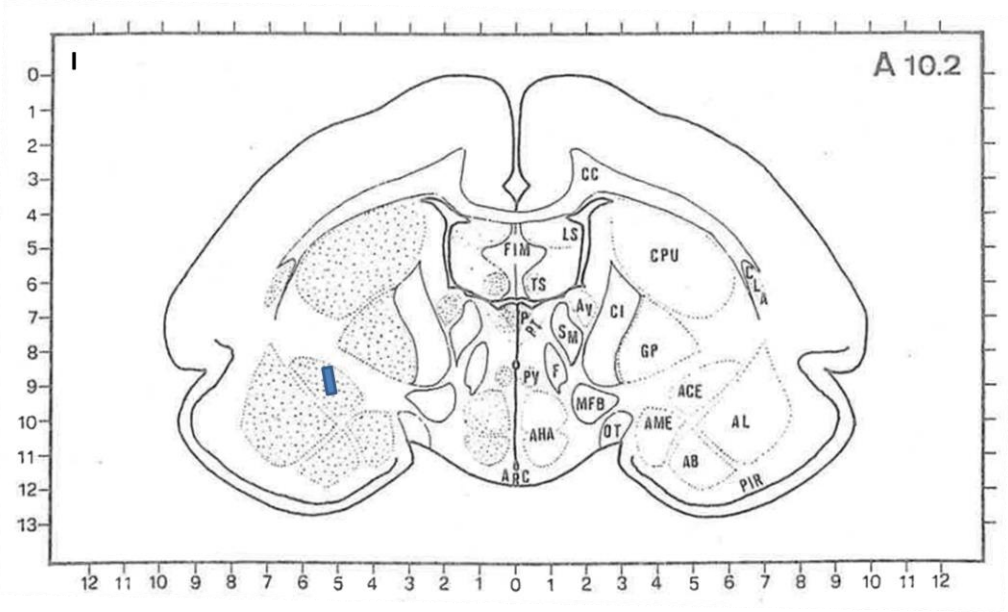
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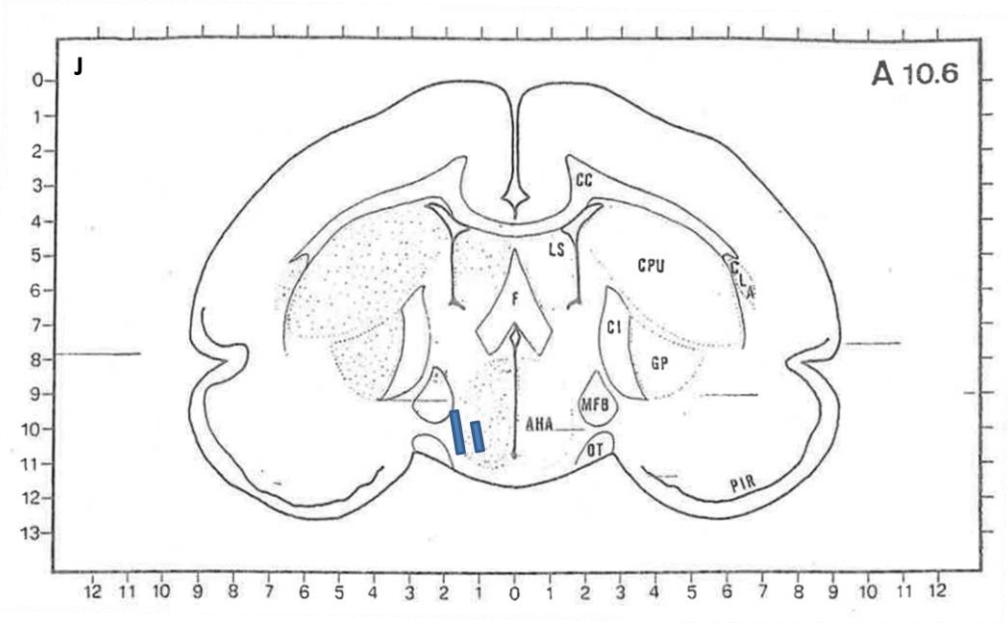
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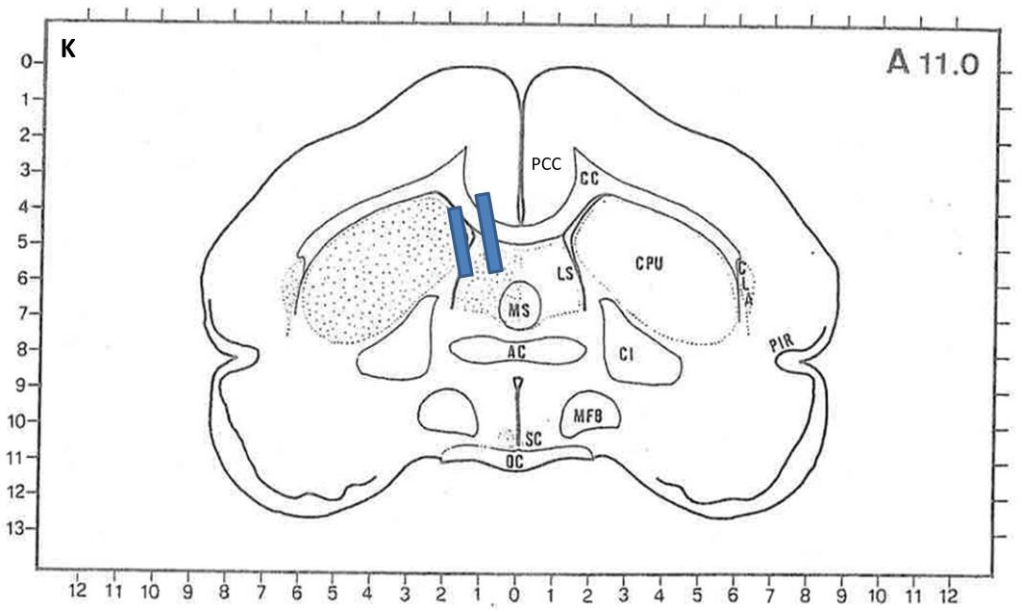
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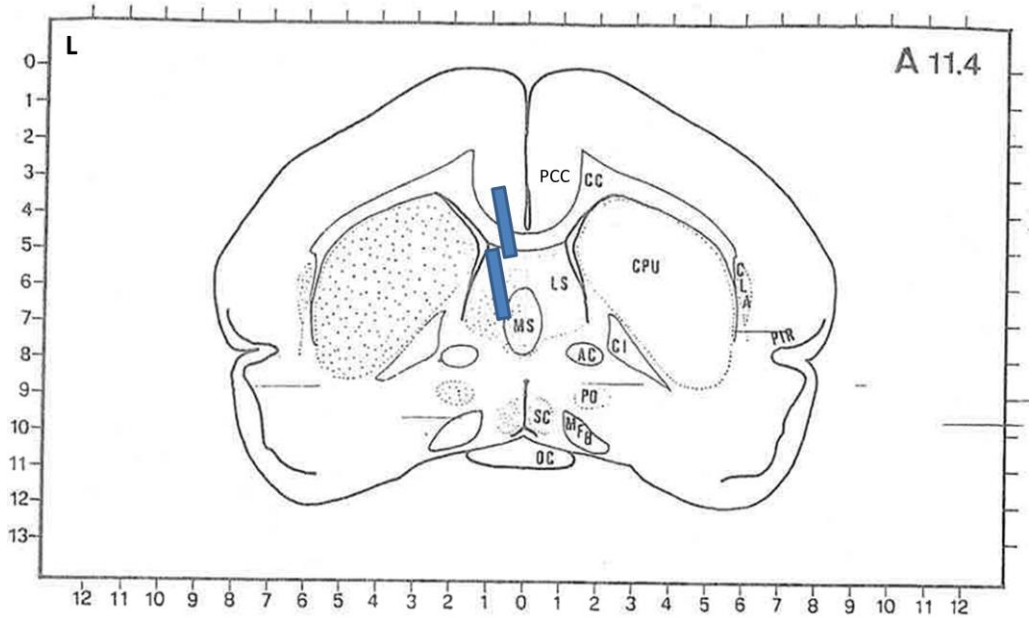
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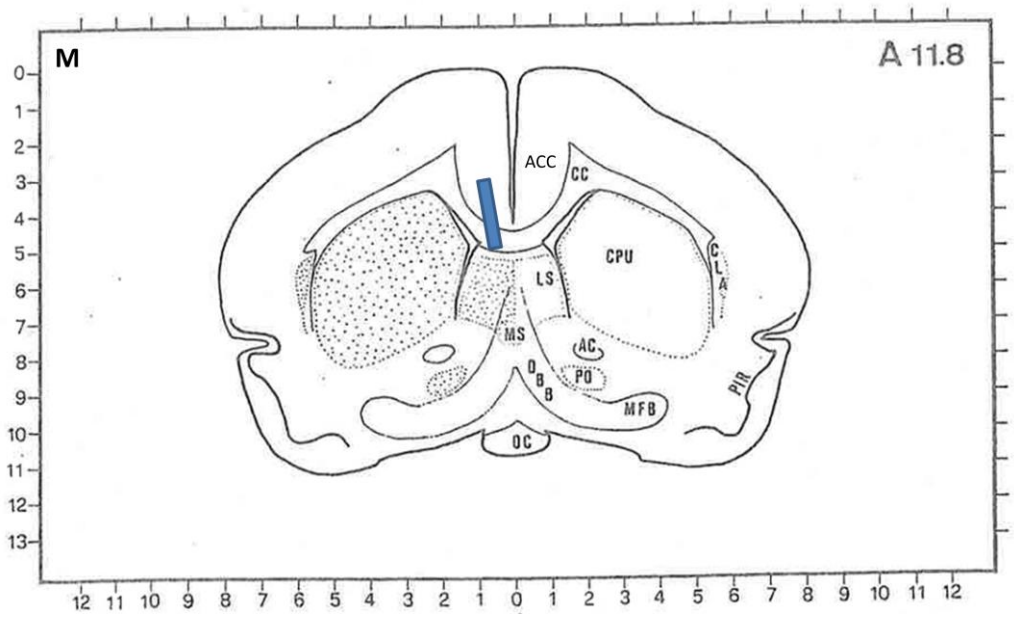
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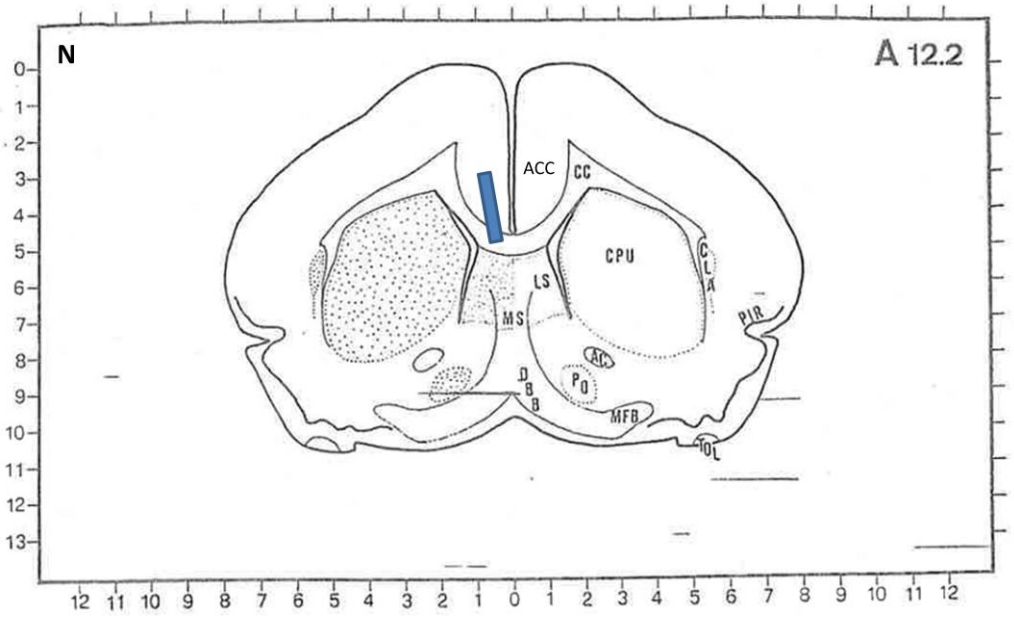
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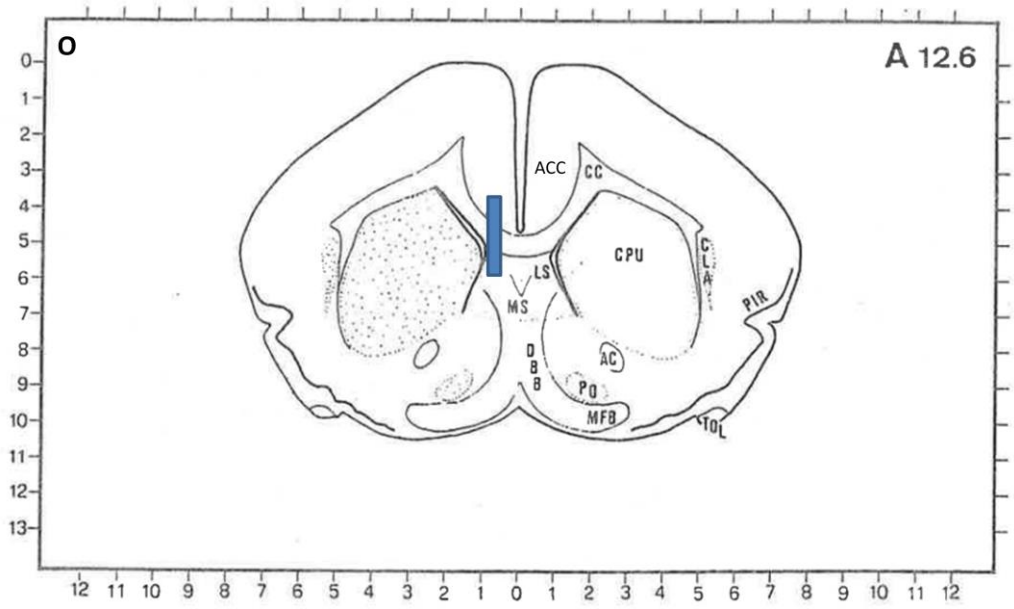
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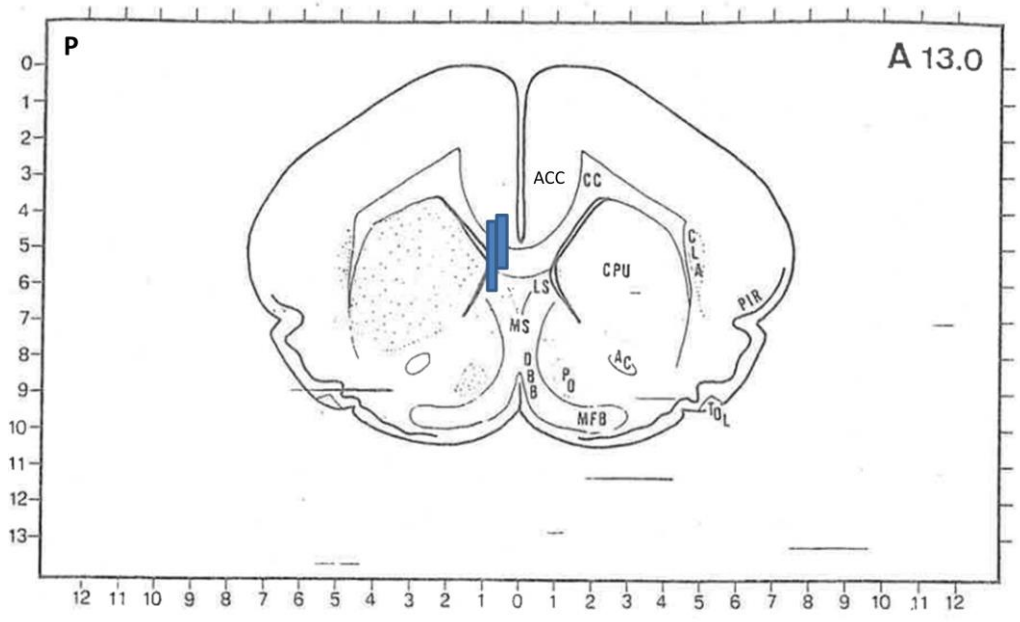
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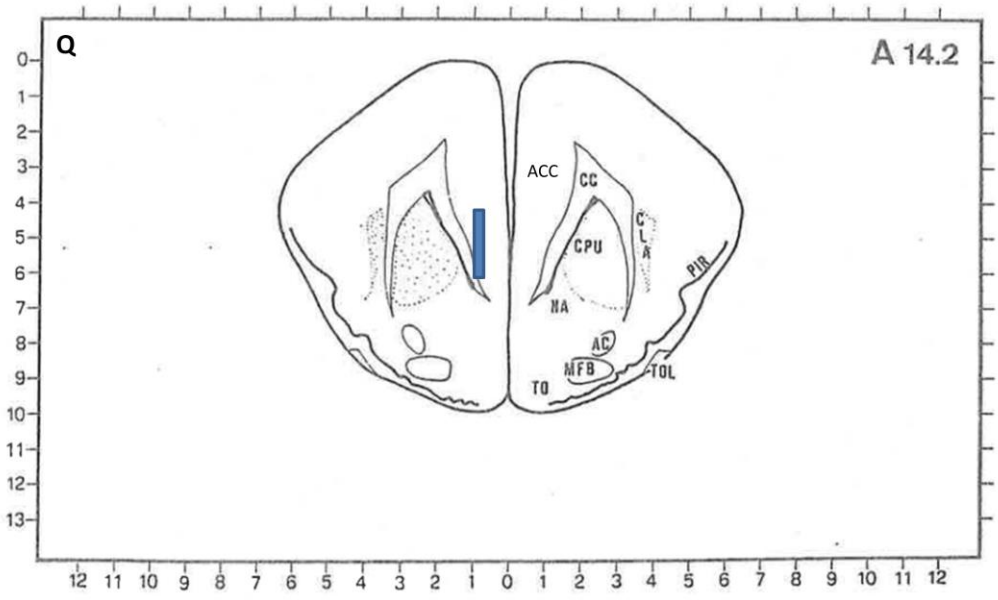
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# Simultaneous Dual Stimulation

The post-stimulus electrically elicited scream/squeal call series could persist for up to thirty seconds. It could also be elicited from numerous spatially separate areas of guinea pig brain; showing similar response to current change in each area. For these reasons, it was proposed that this call is the product of several brain regions working in concert to form excitatory re-entrant loops of neural activity. It was, therefore, hypothesised that subthreshold stimulation delivered simultaneously to two brain areas would yield a call series. These results are presented in section 4.2. In addition to this main aim, the opportunity was presented to investigate a limited number of other interactions. These are presented in sections 4.2 and 4.3.

## 4.1 Bilateral stimulation of two post-stimulus scream/squeal loci

For practical reasons, dictated by access problems with the micromanipulators, in initial experiments electrical stimulation was applied bilaterally in these combinations:

ACC & hypothalamus (n=9 animals), ACC & amygdala (n=7), amygdala & hypothalamus (n=8), medial thalamus & ventral thalamus (n=3), medial thalamus & hypothalamus (n=3), ventral thalamus & hypothalamus (n=6), ventral thalamus & amygdala (n=7).

For all combinations of interactions, RMS values of the dual stimulation call series were significantly higher than either area produced when stimulated individually. Furthermore, the spectrotemporal structure of the dual stimulation call series fit with the classification criteria previously described. Without exception, all of the dual

stimulation interactions were facilitatory and were all areas apparently equipotent in their effect on the other.

As described in section 3.5, repeated stimulation of a scream/squeal locus results in increased intensity of the call series. For this reason, the call intensity data cannot be assumed to be normally distributed. Figure 4-1 shows a boxplot of the interactions between hypothalamus and ACC (10 repeats). The dual stimulation condition produced the scream/squeal call at significantly higher amplitude than each individual area ($p < 0.02$ Wilcoxon signed rank test). At the beginning of the trial both stimuli were subthreshold, each becoming slightly suprathreshold as the trial progressed. Spectrograms of the first repeat are shown in figure 4-2. Examples of call series resulting from high current stimulation of each individual area are shown in figure 4-3. These demonstrate that the dual stimulation effect is akin to increasing the amplitude at a single locus. This supports the hypothesis stated above.

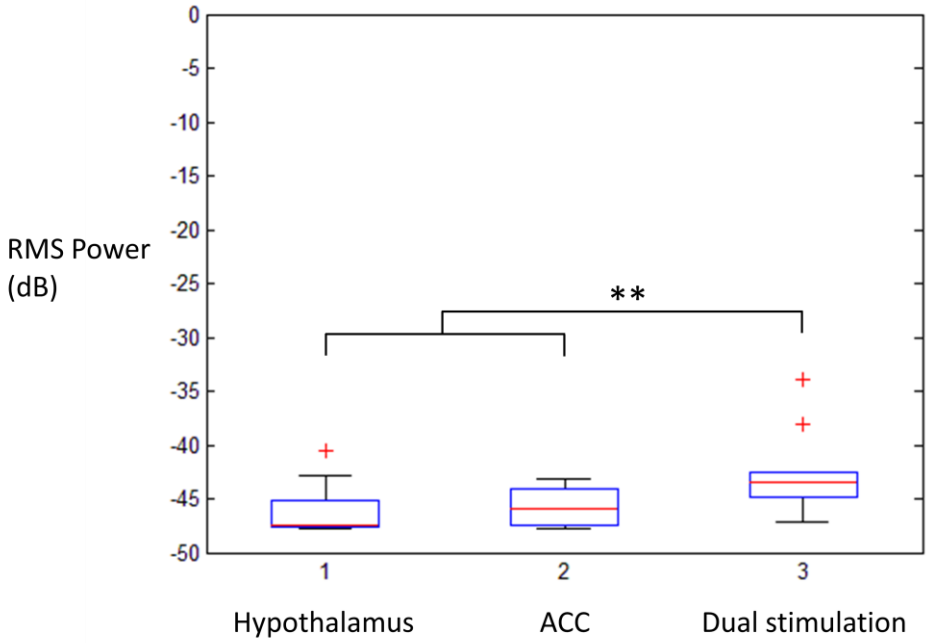



**Figure 4-1** Box plot of RMS values of ten repeats of dual stimulation. Dual stimulation condition is significantly greater than both individual stimulations (** $p<0.02$ Wilcoxon signed rank). Outliers (red+) are greater than 1.5 multiplied by the interquartile range.

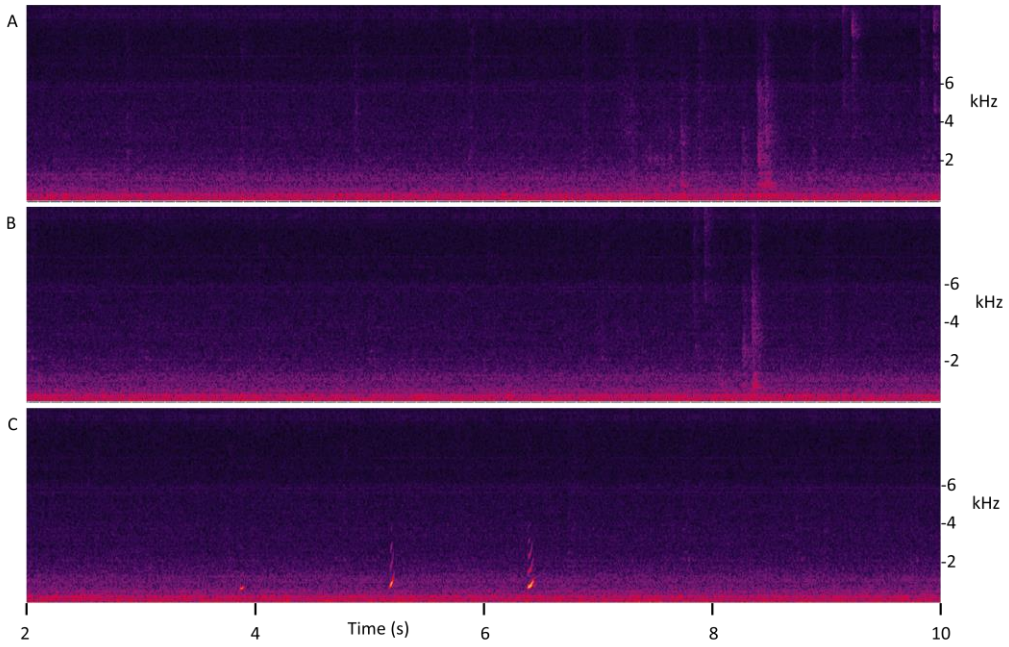



**Figure 4-2** Spectrograms of the first repeat of the dual stimulation test shown in figure 4-1. A) Hypothalamus (70 µA), B) ACC (50 µA), C) Dual stimulation

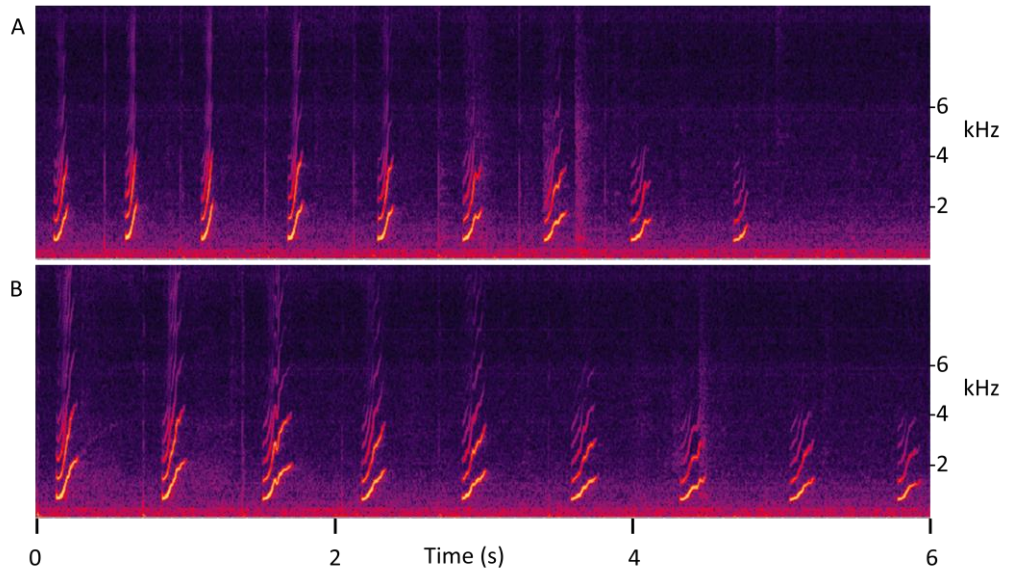



**Figure 4-3** Spectrograms of high current stimulations of the same loci as in figure 4-2. A) Hypothalamus (150 µA), B) ACC (140 µA)

When two areas were stimulated with currents that were slightly above threshold the resulting dual stimulation was always had significantly higher RMS values than when each area was stimulated alone. An example boxplot of such a trial is shown in

figure 4-4. Concurrent stimulation of scream/squeal loci in amygdala and hypothalamus evoked a calls series that was significantly higher in amplitude than each area could produce on its own (10 repeats, $p < 0.005$ Wilcoxon signed rank). As with the previous example, the amplitude of all the calls increased over the duration of the trial, leading to non-normal distributions. Spectrograms of the first repeat shown in figure 4-5.

The results of high current stimulation of each area are shown in figure 4-6. By comparing these to figure 4-5C it is clear that the effect of dual stimulation is similar to the effect of increasing stimulation current in either area.

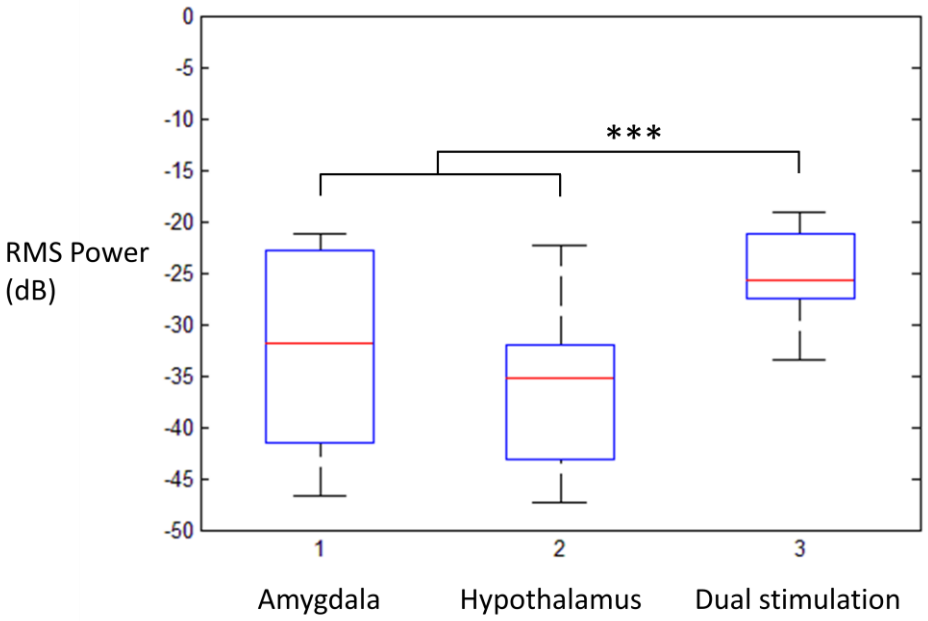



**Figure 4-4** Box plot of RMS values of ten repeats of dual stimulation. Dual stimulation condition is significantly greater than both individual stimulations (*** $p<0.005$, Wilcoxon signed rank).

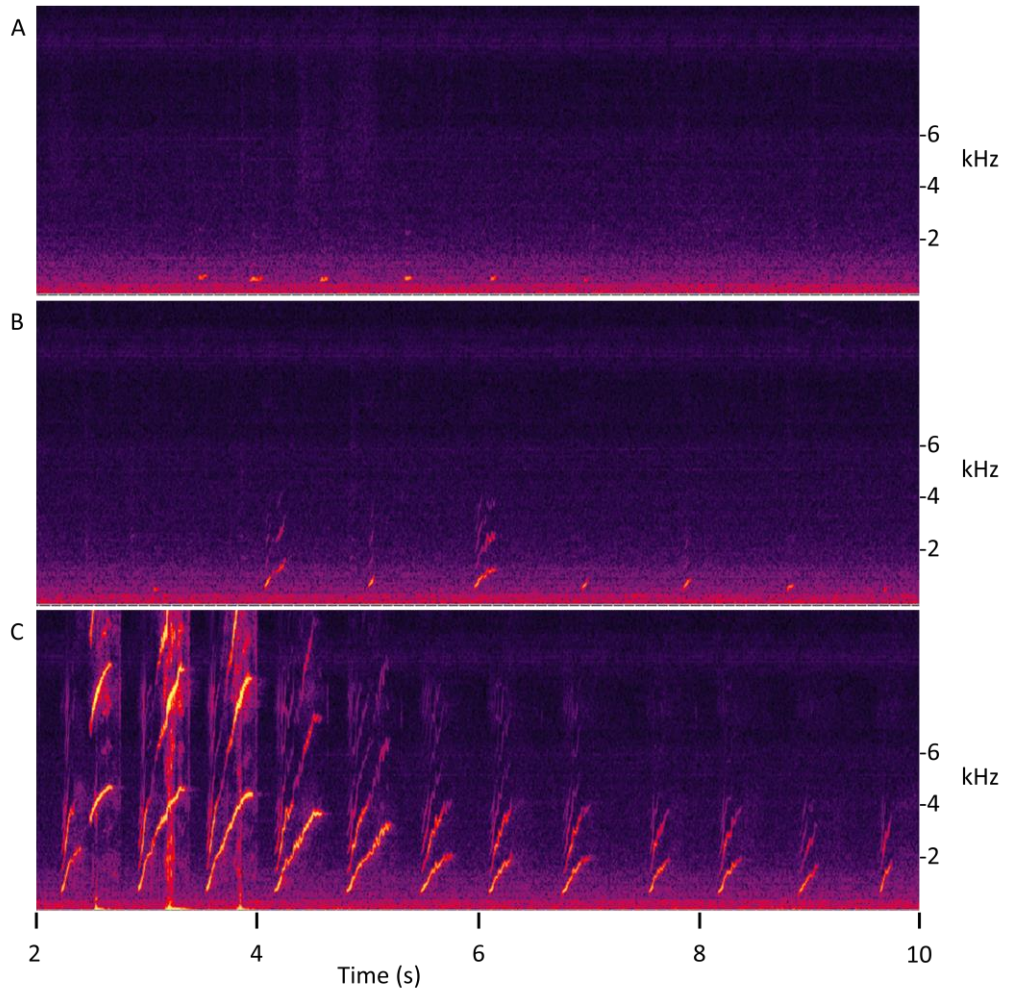



**Figure 4-5** Spectrograms of the first repeat of the dual stimulation test shown in figure 4-4. A) Amygdala (40 µA), B) Hypothalamus (50 µA), C) Dual stimulation

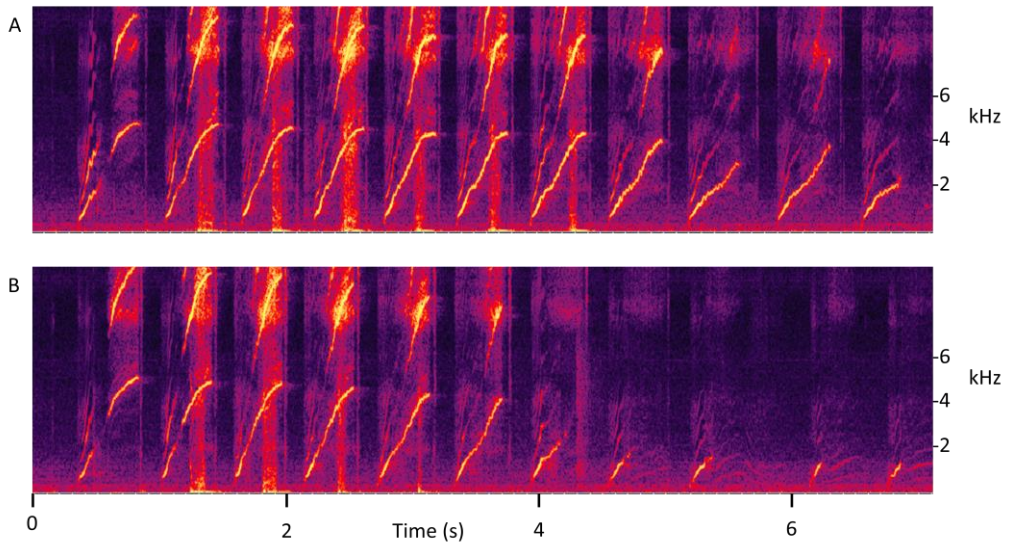



**Figure 4-6** Spectrograms of high current stimulations of the same loci as in figure 4-5. A) Amygdala (200 µA), B) Hypothalamus (200 µA)

**4.2 Dual stimulation of during-stimulus and post-stimulus call loci**

Loci in amygdala which yielded only during-stimulus whine even at high current were stimulated concurrently with post-stimulus scream/squeal loci in the hypothalamus (n=12 animals) and ACC (n=9). Assessment of the dual stimulation effect on whine could not be performed because of the confounding effect of habituation (see section 3.5). Only the effect of the dual stimulation on the post-stimulus squeal series could be assessed. More importantly, these tests provide alternative evidence in support of the claim made by Robinson (1967).

Figure 4-7 shows the amygdala (A) and ACC (B) stimulated individually, dual stimulation is shown in 4-7(C) and dual stimulation after partial habituation of whine is shown in 4-7(D).

On 21 occasions dual stimulation was performed bilaterally between loci in the amygdala producing only during-stimulus whine and loci in ACC (n=12 animals) or hypothalamus (n=9) producing post-stimulus scream/squeal series. An example of one such trial is shown below. Figure 4-7A and B shows the effect of each locus stimulated alone. The first dual stimulation (figure 4-7C) shows a whine followed by an increased intensity scream/squeal series. After the whine had habituated, stimulation of this locus was still able to contribute to the intensity of the scream/squeal (figure 4-7D). In all trials stimulation of the amygdalar whine locus was able to contribute to the post-stimulus scream/squeal series, even after the whine habituated. Figure 4-8 shows a boxplot of RMS values of the post-stimulus time period. (10 repeats, $p < 0.005$).

The amygdalar whine was the only during-stimulus call that could be reliably elicited without being followed by a post-stimulus scream/squeal series. Even so, it is highly probable that nearby scream/squeal loci were stimulated to lesser extent. This is akin

to the subthreshold stimuli described in section 4.2. Indeed, in the example shown, the amygdala locus started to yield a weak post-stimulus call series after several repeats.

Over the ten repeats of the trial, the during- and post-stimulus calls responded with habituation and facilitation, respectively. This finding supports, by different means, a previous assertion that production of these two classes of calls is mediated by separate neural circuitry (Robinson, 1967).

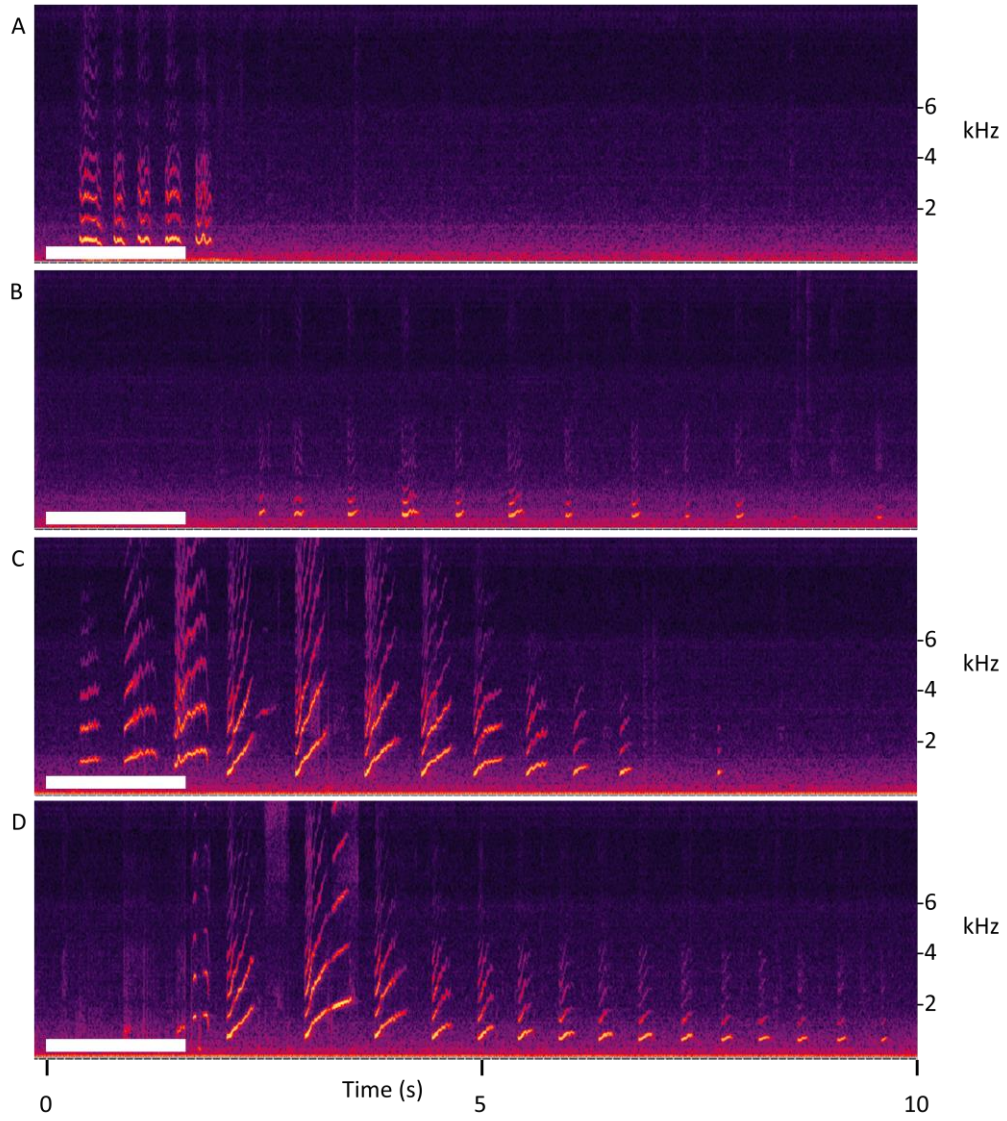



**Figure 4-7** Dual stimulation of an amygdalar during-stimulus whine with an ACC elicited post-stimulus scream/squeal series. A) Amygdala only stimulation (100 µA), B) ACC only stimulation (50 µA), C) First dual stimulation, D) Second dual stimulation.

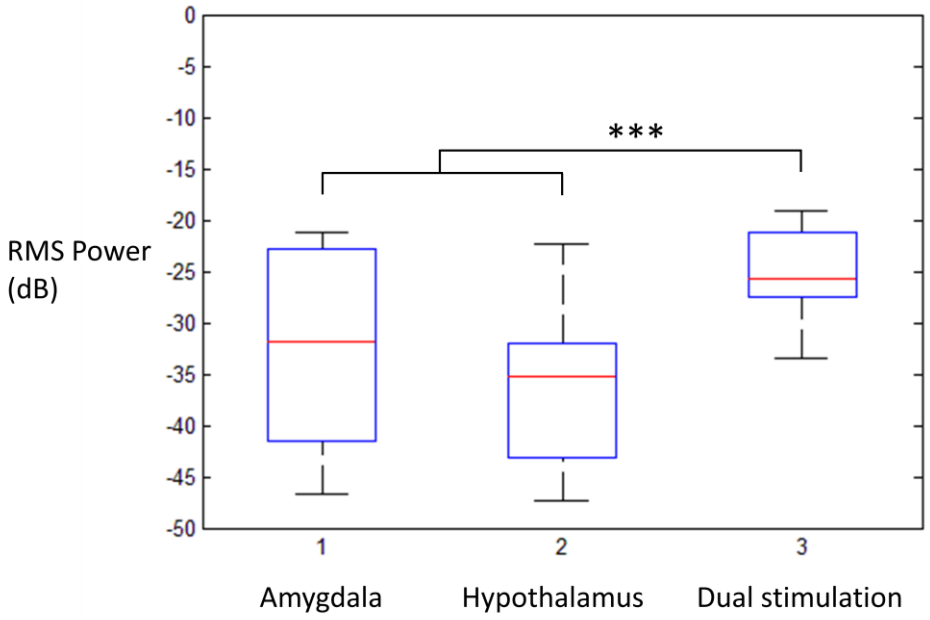



**Figure 4-8** RMS values of the post-stimulation period following dual stimulation of amygdalar whine and hypothalamic scream/squeal (10 repeats). The dual stimulation condition is significantly greater than both individual stimulations (*** $p<0.005$ Wilcoxon signed rank).

An alternative explanation for this phenomenon is that both effects (habituation of whine and enhancement of scream/squeal) are a result of stimulation-related changes in the same neural circuitry. For this reason, the progression of these two effects were assessed over the time course of the dual stimulation trial.

Data were analysed from dual stimulation of during-stimulus whine in amygdala and post-stimulus scream/squeal in hypothalamus in 6 GPs. Figure 4-9 shows the percentage change in amplitude (RMS) against repeat number, normalised to the 1$^{st}$ measurement. The during-stimulus values are inverted to allow for easier comparison. The overlapping error bars suggest that the progressions of the two effects are not significantly different yet, they are not sufficiently similar as to support the hypothesis stated above. Alternative experimental methods would be

required to further investigate this hypothesis. Such methods are discussed in section 7.1.0.

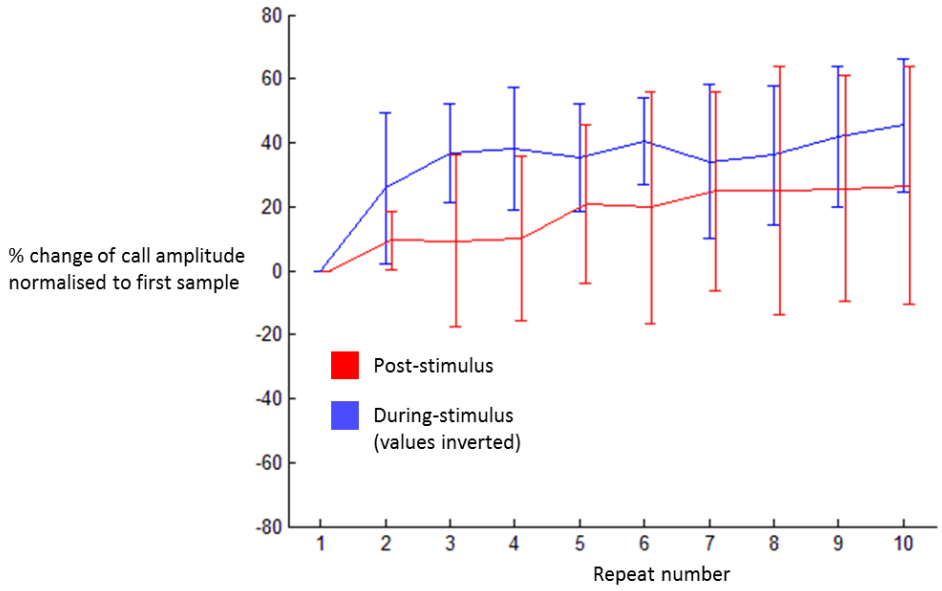



**Figure 4-9** Percentage change in amplitude of during-stimulus whine (values inverted, blue) and post-stimulus scream/squeal (red). Values normalised to the 1st measurment. Error bars ± 1 standard deviation.

### 4.3 Dual stimulation of two during stimulus vocal production loci

With the exception of whine elicited from the amygdala, during-stimulation loci were rarely found. It was even rarer, therefore, that two could be located at the same time in order investigate the effect of concurrent dual stimulation.

There were only three such occurrences. Two of these involved the same call type elicited from different nuclei and on different cerebral hemispheres. These were: whine from hypothalamus and amygdala; and chut from ACC and hypothalamus. In both these cases the call produced from dual stimulation matched the

spectrotemporal properties of both the individual stimulations, with no differences in latency. There were also no apparent differences in intensity. Statistical analysis was not performed because any slight variation could not be separated from the confounding effect of habituation. This suggests that, in these instances, there is no interaction for this call type at these locations.

On one occasion an electrode array aimed at the medial hypothalamus crossed the midline and was able to elicit a chut from the opposite hemisphere. This, then, was stimulated concurrently with an amygdalar whine, giving two different call types from two different nuclei in the same hemisphere. Again, due to the rapid habituation of all during stimulus vocalisations, statistical analysis could not be performed.

Figure 4-10 shows spectrograms from this trial. 4-10A shows the whine elicited from amygdala and 4-10B shows the chut elicited from hypothalamus. The abnormal spectrotemporal structure of the chut is due to the guinea pig having an obstruction in its throat that was only discovered and removed subsequent to the trial. The first dual stimulation is shown in 4-10C, here the whine starts with the same latency as in the single stimulation, it is then suppressed, presumably by the same neural activation that led to the chut. By the second dual stimulation (figure 4-10D) the hypothalamic chut is almost completely suppressed yet there is still a suppressive effect as shown by the truncated whine.

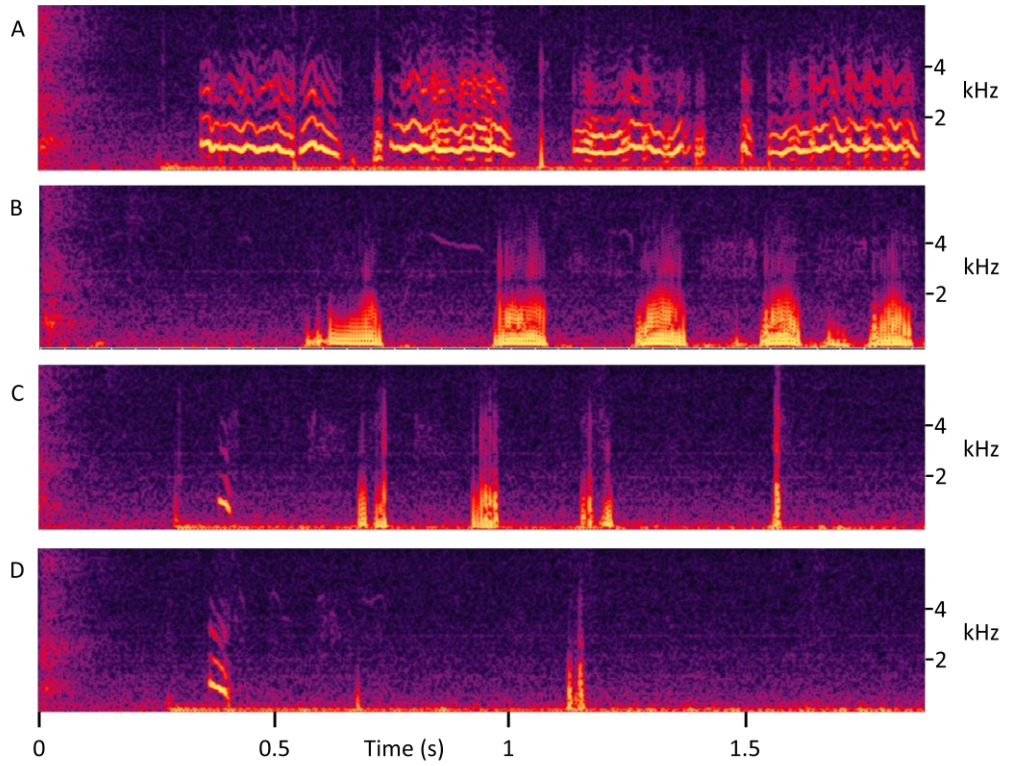



**Figure 4-10** Dual stimulation of two during-stimulus loci. A) amygdalar whine (100 µA), B) hypothalamic chut (120 µA), C) concurrent stimulation of loci A and B at the same current, D) dual stimulation following habituation of the hypothalamic chut.

### 4.4 Summary

Owing to the diffuse nature of scream/squeal loci, the intended dual stimulation tests were less informative that was hoped. The other two tests were performed in an ad hoc manner yet yielded some interesting results. In particular, dual stimulation of two during-stimulus loci has the potential to provide valuable results in future, as part of a hypothesis-driven experiment.

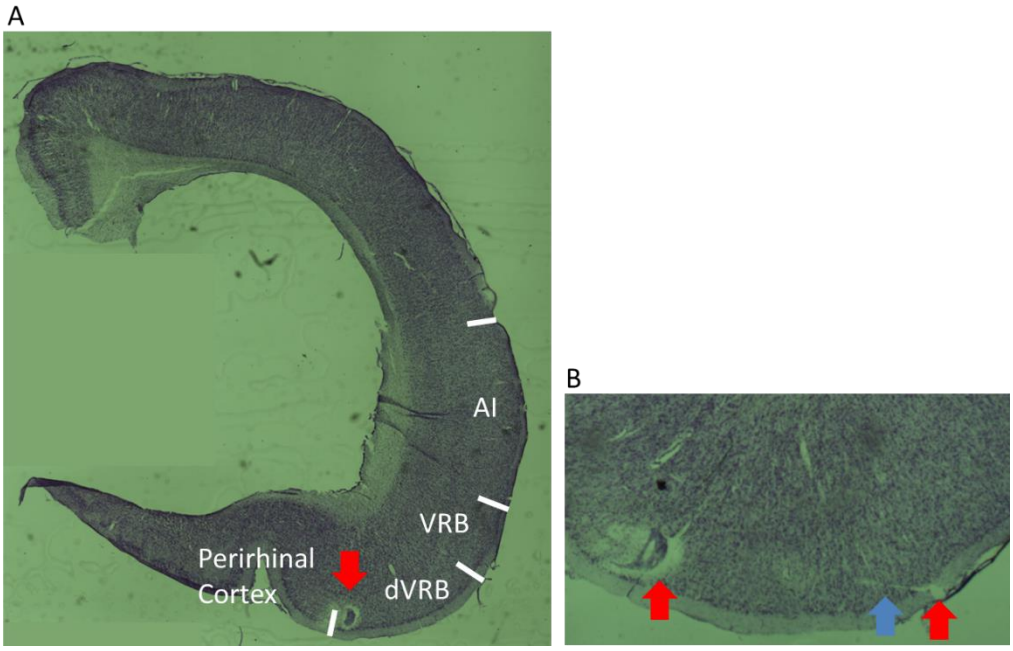
# A new auditory sensitive cortical area

The initial electrode placement aimed to record from the ventral portion of VRB so as to be sure of being in VRB rather than A1 (see figure 1-7). The physiological characteristics, however, did not match those previously documented for VRB.

It was previously thought that GPs, along with all nonprimate species, do not have auditory regions further ventral than VRB (Redies et al., 1989; Wallace et al., 2000). The new cortical region I have discovered contradicts these previous studies. In this work it is referred to as deep ventrorostral belt (dVRB). An argument for its distinction from the known VRB area is presented below. A total of 270 dVRB single-neurons were recorded from 8 animals.

## 5.1 Anatomy and histology

In one animal electrolytic lesions were made at the top and bottom of a successful recording track in dVRB. Figure 5-1 shows the locations of the lesions in an area of neocortex further ventral to VRB. Between these two lesions, auditory sensitive neurons were recorded every 100 μm on at least one of the four electrodes in the array. Based on cranial landmarks, the electrophysiological recordings for the other s




even GPs were also in this brain area.

**Figure 5-1** Histological verification of dVRB. A) Coronal section of the whole of the right guinea neocortex showing location of the deep lesion in dVRB B) Enlarged image from the adjacent section showing lesions at the top and bottom of the track. Lesions are indicated by red arrows and the track mark by a blue arrow. The orientation of this section has been rotated in a clockwise direction by about 50°as the electrode was inserted at an angle of 40° to the vertical and not in the horizontal plane as implied here.

### 5.2 Physiological responses to simple auditory stimuli

Guinea pig AI and VRB respond well to auditory presentation of puretone sine waves, and are tonotopically organised (Grimsley et al., 2012; Wallace et al., 2000). The 270 dVRB neurons were probed with a similar range of puretone stimuli (see section 2.8.1 for details). In contrast to AI and VRB, none of the dVRB neurons responded to puretone stimuli.

AI and VRB also respond consistently to broadband clicks (Grimsley et al., 2012; Wallace et al., 2000). Again in contrast, only 13 dVRB neurons responded to a

broadband click. Using puretones at CF and broadband clicks, Wallace (2000) recorded the onset response latency on 98 VRB neurons and reported a range of 15 – 44 ms. Of the click-responsive dVRB neurons, none showed high temporal precision to the onset of the click and a wide range of latencies, distinct from that of VRB, was observed. Figure 5-2 shows these latencies, as measured from peak response time, and figure 5-3 shows three example neurons from that group. Of particular note is that neurons A and C were recorded from adjacent electrodes on the same track and were less than 300 µm apart. A latency of 30 ms would be suggestive of VRB. However, this neuron's proximity to another with 100 ms latency, coupled with the lack of pure tone responses throughout the whole electrode track, means it is not in VRB.

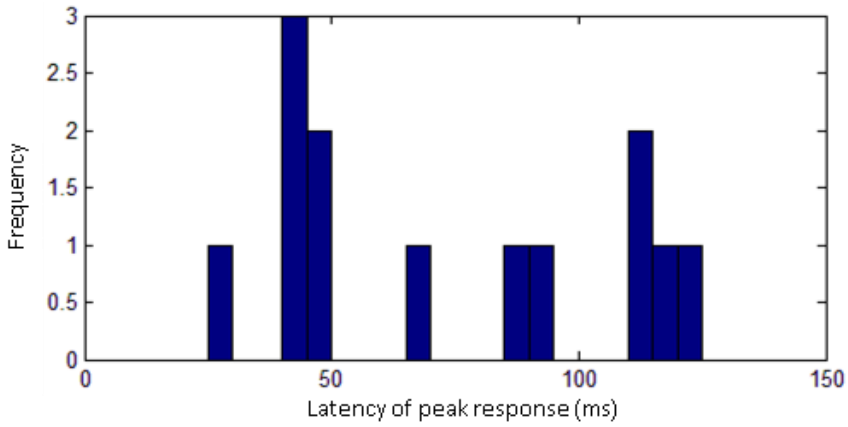



**Figure 5-2** Histogram (bin size 5 ms) of the range of latencies of dVRB neurons that responded to broadband clicks.

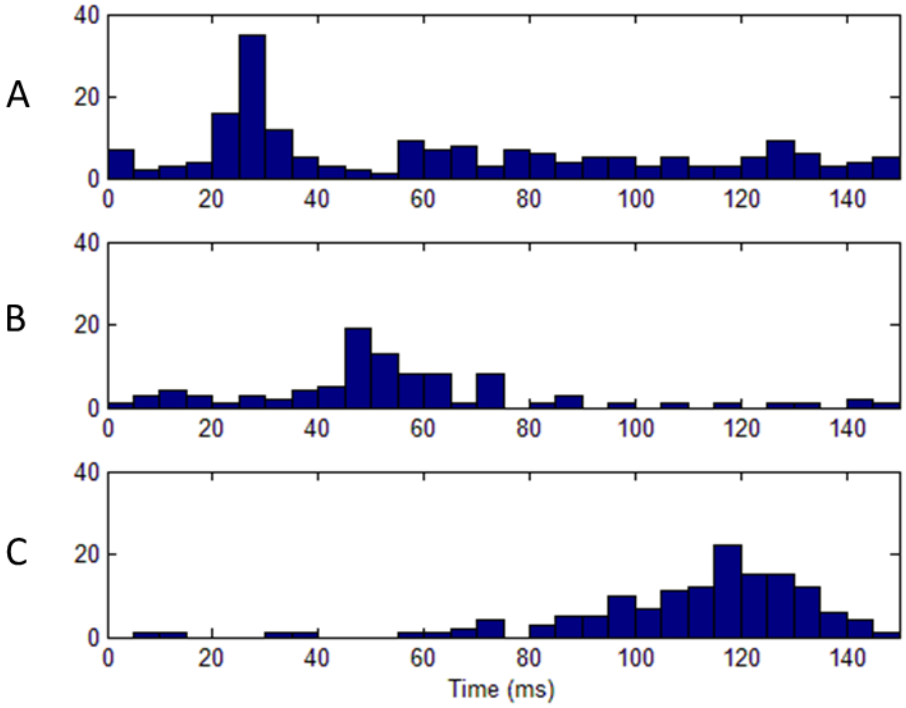



**Figure 5-3** Post stimulus time histograms (bin size 5 ms) of three example neurons showing a response to broadband click in dVRB.

### 5.3 Physiological responses to complex auditory stimuli

All the neurons recorded in dVRB gave strong responses to one or more of the battery of complex auditory stimuli: conspecific vocalisations and white noise.

The primate superior temporal gyrus (STG) encompasses both belt and parabelt regions in primates. Single-neuron studies in macaque, squirrel monkey and marmoset have implicated all areas of the auditory STG in the ability to discriminate between conspecific vocalisations (Ghazanfar and Hauser, 2001; Recanzone, 2008; Wang et al., 1995). Similar evidence has been acquired in guinea pig VRB which is ventral to AI (Grimsley et al., 2012). The present study did not use the full repertoire of GP vocalisations. The fact that area dVRB also responds to guinea pig vocalisations suggests that a similar study, with the full range of calls and analysis based on information theory, would be informative.

## Audio-Vocal (AV) Interactions

During phonation the vocal production system interacts with the auditory system to communicate the expected sensory consequences of the motor output. These are known as audio-vocal (AV) interactions and allow one to discriminate between self-produced and external sounds. BA is known to be involved in vocal production and, in GP, the whine call can be elicited from here using electrical stimulation. I wanted to investigate whether BA activation could modify the auditory cortical representation of conspecific vocalisations.

Previous AV studies have used combined microstimulation and electrophysiology (Alexander et al., 1976; Müller-Preuss et al., 1980). These focused primarily on the changes to spontaneous firing in auditory cortical neurons that could be produced by stimulating vocal production areas. Furthermore, the stimulation produced artefacts in the neural recordings so they were restricted to observing the effects immediately following cessation of the stimulation. Here, I was able to eliminate this artefact, thus allowing concurrent presentation of both auditory and electrical stimuli (see section 2.5.2). This simultaneous activation of both the vocal production and auditory systems is more relevant to the physiology in a spontaneously vocalising animal.

Initially, a whine call was elicited by electrical stimulation (100 µA continuous 10 ms sine wave for 800 ms) of BA whilst the GP was lightly anaesthetised. At the deeper level of anaesthesia that was maintained during the electrophysiological recordings, this same electrical stimulus would *not* elicit a call. Single-neuron responses to auditory presentation of eight vocalisations were recorded both with and without concurrent electrical stimulation in BA.

For each auditory stimulus in each neuron a normalised response modulation index (RMI) value was calculated. Positive RMI values indicate that electrical stimulation enhanced the neural response to a vocalisation; negative RMIs indicate that electrical stimulation caused suppression of the neural response; N/A indicates that a neuron either did not respond or, showed no significant change in response as a result of electrical stimulation.

### 6.1 Electrophysiological recordings from two areas of auditory cortex

A total of 56 neurons were recorded in primary auditory cortex (AI) in 5 animals. Characteristic frequencies (CF) ranged from 6 – 9 kHz. Latencies to presentations of broadband clicks and CF pure tones were between 10 and 15 ms. These values were in the range expected for AI and demonstrated that the units were not in VRB, where the latencies are all 16 ms or above (Wallace et al., 1999). In one experiment the location of the recording site in AI was also confirmed by histology.

270 auditory sensitive neurons were recorded in dVRB. Their unusual responses to simple auditory stimuli were discussed previously (see section 5).

### 6.2 AV interactions in AI

The majority, 52 of 56, AI neurons gave a significant response to two or more of the auditory stimuli. Concurrent BA stimulation had a variety of effects within individual neurons, depending on the call types. Figure 6-1 shows an example of a neuron that gave significant responses to 4 of the complex auditory stimuli. These responses were modified by concurrent electrical stimulation of the BA vocal production region.

The nature of the modification, whether suppressive or enhancing, depended on the call type. The neural response, therefore, represents a complex interaction of both the auditory and electrical stimuli.

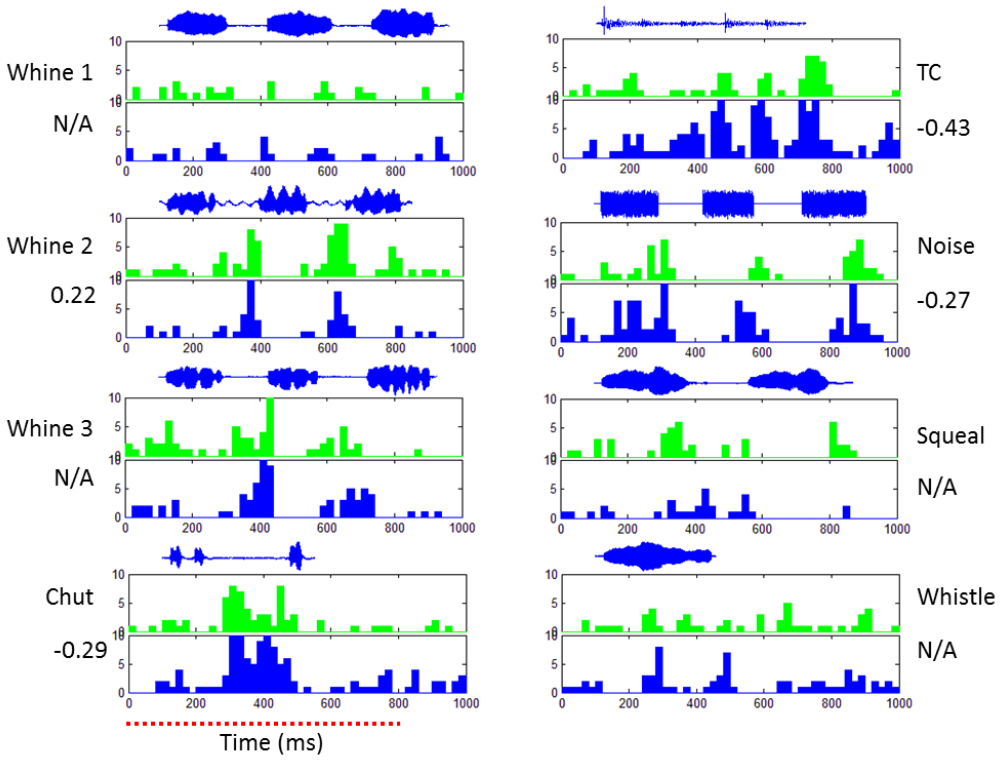



**Figure 6-1** AI neuron (CF 8 kHz, latency 10 ms) showing a variety of enhancements and suppressions of auditory responses by electrical stimulation of BA depending on call type. For each subplot the waveform is shown at the top, with histograms (bin size 20 ms) showing pooled responses of 20 repeats to auditory only stimulation (blue) and auditory and electrical stimulation (green). The electrical stimulation period was 0 – 800 ms (red dash line). The call types and RMI values are shown to the side.

Figure 6-2 shows histograms of the RMI values for each call type across the 56 AI neurons. Neurons did not give significant response modulations to all auditory stimuli, hence the varied sums of the histograms. There appears to have been no selective modulation to any single call type or group of call types. The distributions in each histogram show a slight, yet distinct positive shift. Therefore, across the

population of AI neurons, the response to each call types is slightly enhanced by BA stimulation.

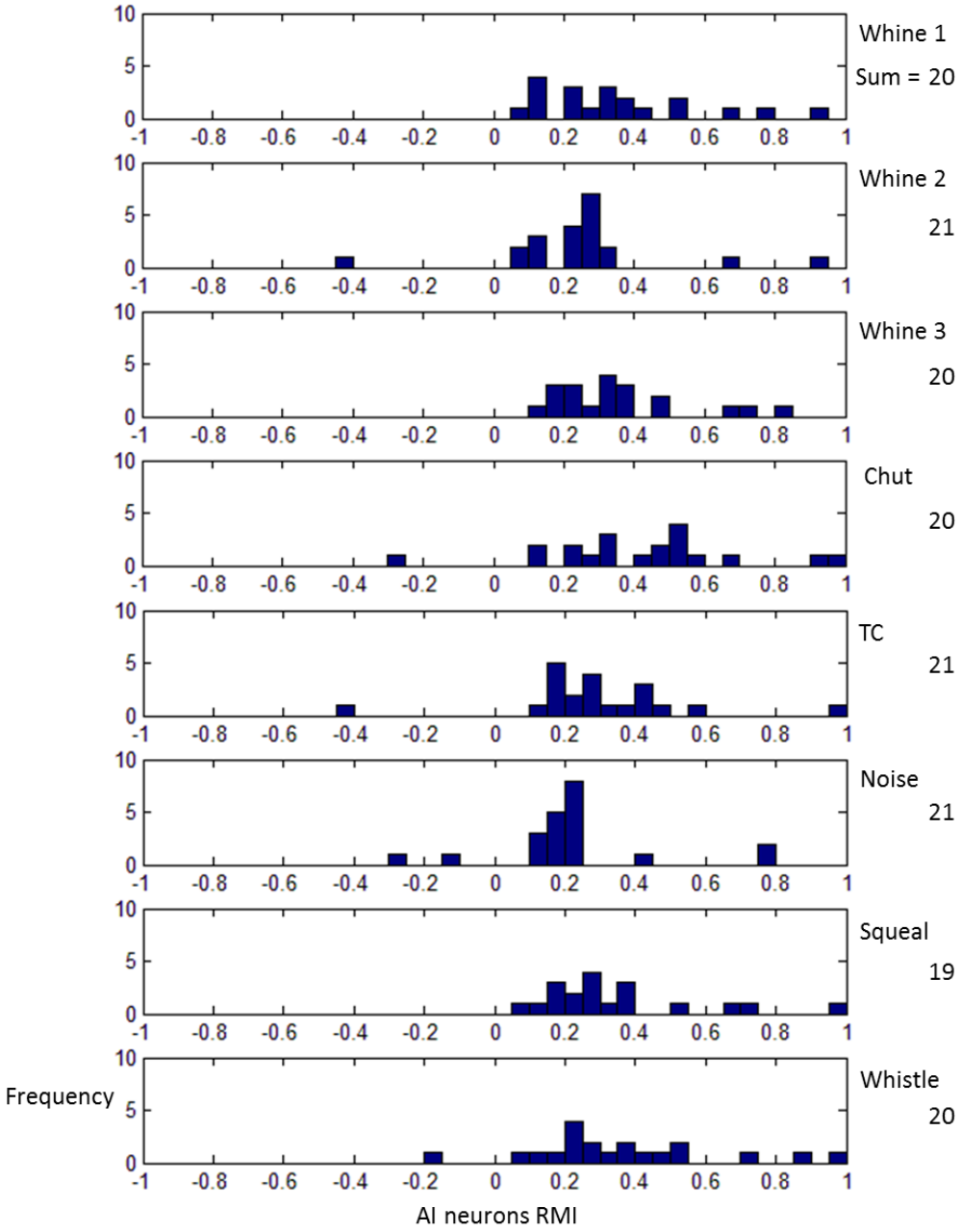



**Figure 6-2** Histograms of the response modulations of 56 AI neurons to each auditory stimulus. Bin size is 0.05 RMI. The total number of neurons giving significant responses are shown under each call type.

The response modulations to the different call types within individual neurons were varied and no discernable patterns could be found. No correlations were found between a neuron's CF and its response characteristics. The same has been reported by AV research in primates (Eliades and Wang, 2005). Furthermore, the responses to the three whine calls were neither distinct from the other call types nor from each other.

Figure 6-3 shows the RMI values for each call types in each neuron. The first 5 neurons in figure 6-3 A significantly modified just one of the auditory stimuli, with a variety of effect sizes. All other neurons in this group show a variety of strengths of enhancement, depending on call types, and a variety of ranges.

In the majority of neurons showed only positive RMIs. Of these, 4 had significant RMI values for only one call type. The other 40 in this group showed a variety of response strengths and a variety of RMI ranges, although with no apparent pattern. Three neurons showed only suppressed responses. In each of these cases, only one call response was significantly modified. Just one neuron showed a mixture of enhancement and suppression of auditory responses. Eight neurons showed no significant modification to any call response.

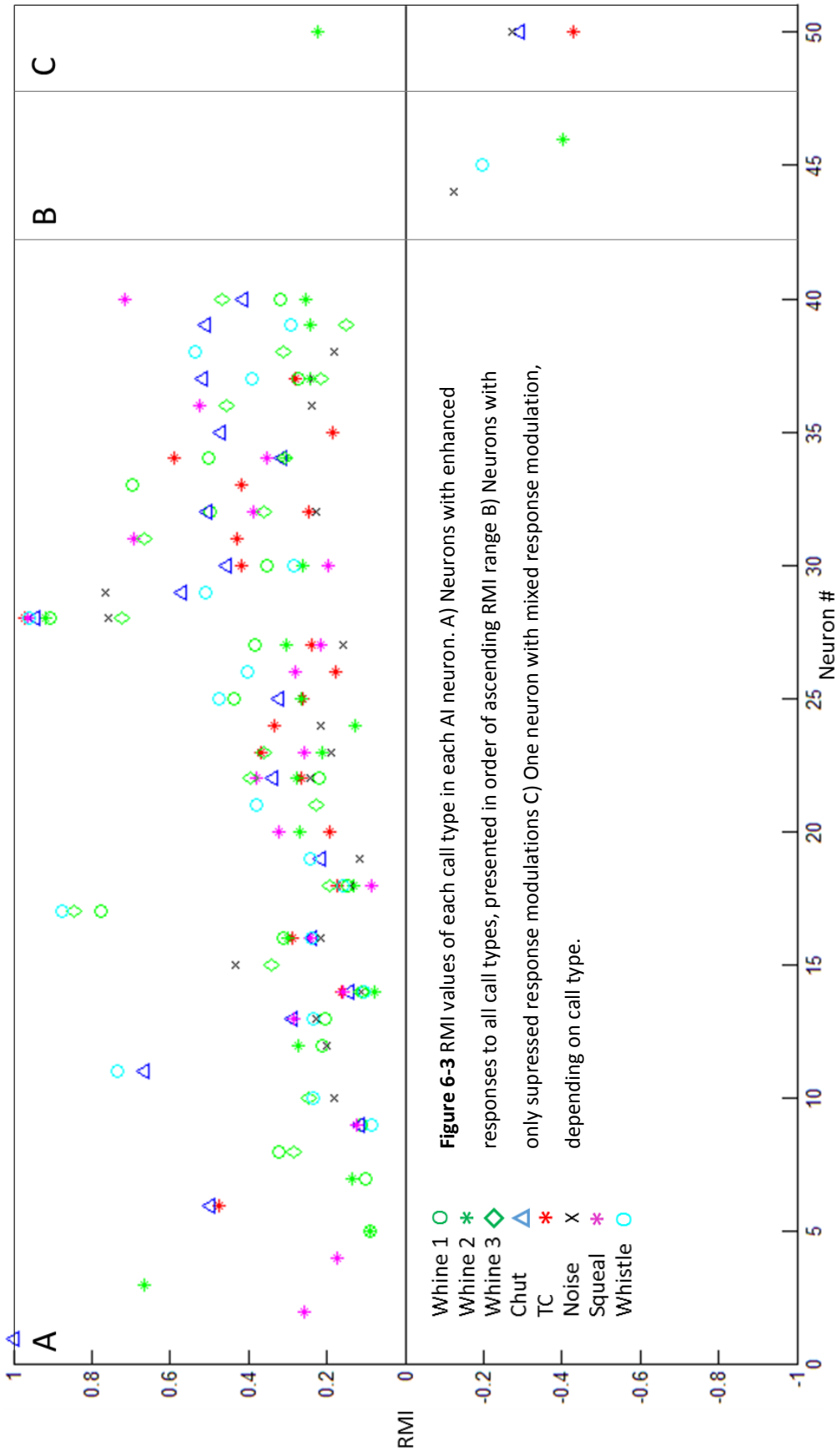



**Figure 6-3** RMI values of each call type in each AI neuron. A) Neurons with enhanced responses to all call types, presented in order of ascending RMI range B) Neurons with only supressed response modulations C) One neuron with mixed response modulation, depending on call type.

### 6.3 The effects of amygdala stimulation in AI

In order to mimic the sequence of neural activation in a spontaneously vocalising animal, the auditory stimuli were presented with a latency of 100 ms with respect to the onset of electrical stimulation. During the initial time period it was possible to observe the effect of the electrical stimulation alone in the AI neurons. RMI values were calculated from pooled spike counts in all the trails in the initial 10 – 100 ms period. The significance of these values was determined using the same randomised distribution method as described in section 2.9.4.

Of 56 AI neurons, 33 showed significant electrical-only modulation. Their distribution is shown in figure 6-4. All 33 show an increase in spontaneous spiking caused by the BA stimulation. Furthermore, there is a broad distribution of the strength of this enhancing effect.

AV research in spontaneously vocalising marmosets reported changes in firing rates of AI neurons up to 400 ms in advance of vocal production (Eliades and Wang, 2005), and I and others have shown ~400 ms latencies for electrically elicited vocalisations (Jürgens, 1998). It is reasonable, therefore, to expect changes in spontaneous activity caused by BA stimulation. The marmoset study, however, reported a higher proportion of AI neurons that showed reduced activity prior to vocal onset. This is in contrast to the results shown in figure 6-4. The fact that the vocal production system was activated in an artificial way is a likely cause of this disparity.

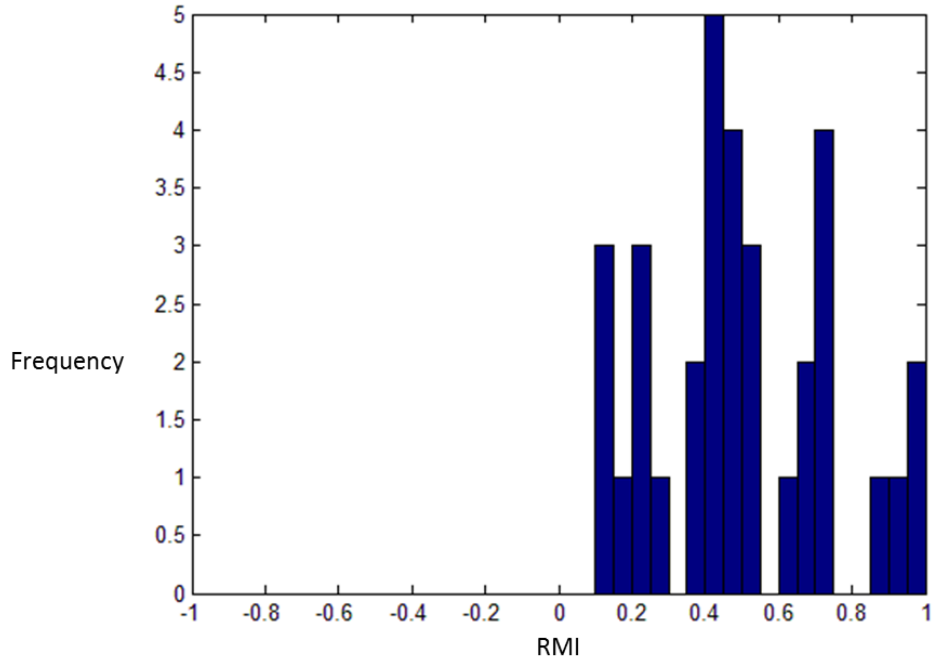



**Figure 6-4** Electrical stimulation of BA causes increases in spontaneous spiking activity in the majority of AI neurons. Histogram of RMI values for the 33 significantly modulated AI neurons, calculated from pooled spike counts in the period 10 -100 ms. Bin size 0.05 RMI.

In addition to onset latency, my microstimulation study showed that an elicited vocalisation would persist up to 200 ms after cessation of the electrical stimulation (see figure 4-9), and similar offset latencies have been reported in squirrel monkey (Jürgens, 2002). In spontaneously vocalising marmosets, some AI neurons displayed modulatory effects of self-produced vocalisation to the end of the vocal phrase (Eliades and Wang, 2003). This implies that there is a persistent AV interaction to account for this time difference, and that post-stimulus effects of BA stimulation would result in modified spontaneous firing in AI neurons.

In the trials for the call types chut and whistle, the period 850-1000 ms was used to investigate any persistent effects of the microstimulation. This time period was over 400 ms after the termination of auditory stimuli, and 50 ms after the end of the

electrical stimulation, so changes in spontaneous firing could be assumed to be caused by electrical stimulation alone. RMI values were calculated from the pooled spike counts to compare the effect of electrical stimulation alone in the post-stimulation time period. Only two neurons showed significantly modulated responses due to the sustained effect of BA stimulation. Both were suppressed, with RMI values of -0.56 and -1. In contrast to the previous figure, there is scant evidence of any persistent effects of stimulation.

**6.4 AV interactions in dVRB**

In 8 animals, 270 auditory sensitive dVRB neurons were recorded. Of these, 257 gave significant response to multiple call types. The responses of dVRB neurons to auditory stimuli were also modulated by concurrent electrical stimulation of BA. Furthermore, the modulatory effect was dependent on call type. As with AI neurons, dVRB single-neural activity represents a complex interaction between both auditory and electrical stimulation. Figure 6-5 shows an example neuron from dVRB with significant enhancement of auditory response to 5 call types caused by concurrent electrical stimulation of BA.

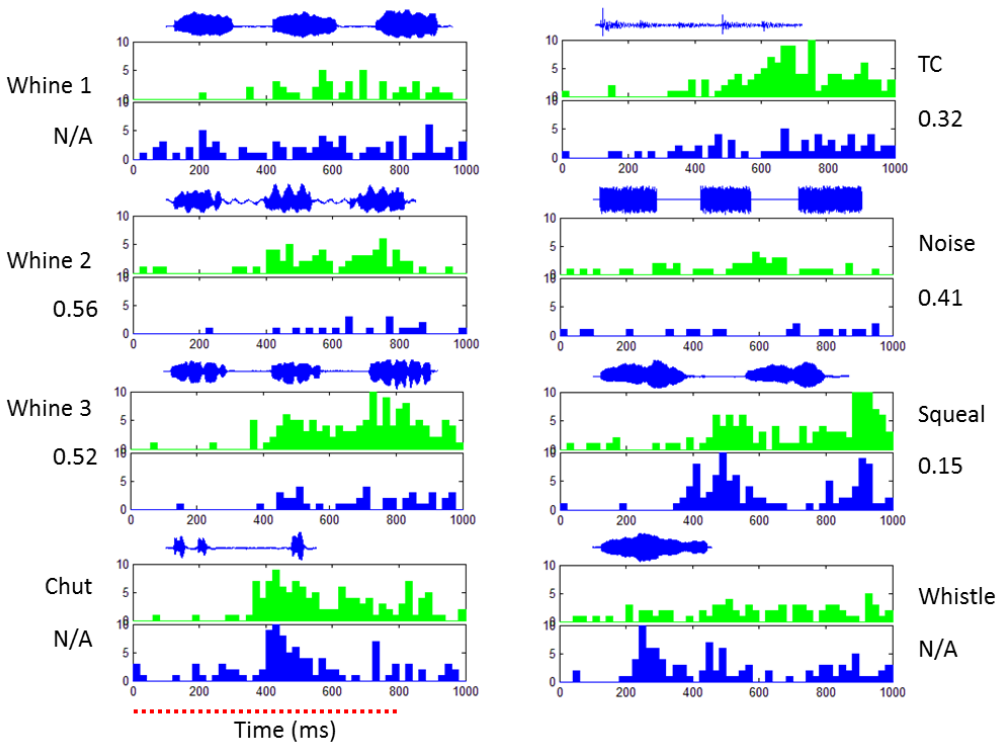



**Figure 6-5** dVRB neuron showing a variety of strengths of enhancement of auditory responses by electrical stimulation of BA depending on call type. For each subplot the waveform is shown at the top, with histograms (bin size 20 ms) showing pooled responses of 20 repeats to auditory only stimulation (blue) and auditory and electrical stimulation (green). The electrical stimulation period was 0 – 800 ms (red dash line). The call types and RMI values are shown to the side.

Figure 6-6 shows histograms of the RMI values of these neurons divided by call type. As with AI, there appears to have been no selective modulation to the response to any single call type or group of call types. The distributions in each histogram show stronger positive distribution than AI. Therefore, across the population of dVRB neurons, the response to each call type is strongly enhanced by BA stimulation.

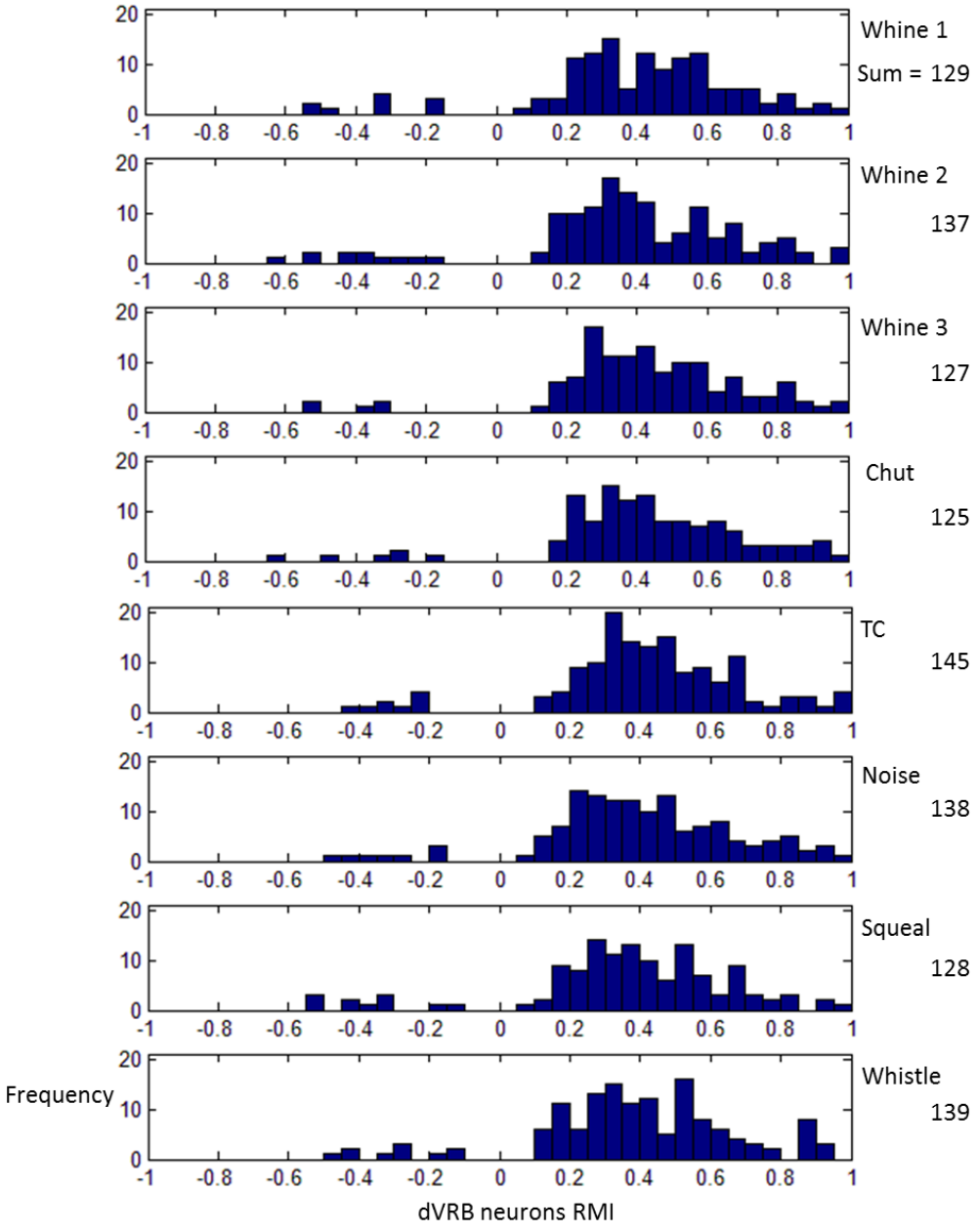



**Figure 6-6** Histograms of the response modulations of 270 dVRB neurons to each auditory stimulus. Bin size is 0.05 RMI. The total number of neurons giving significant response modulations are shown under each call type.

As with the AI neurons, the response modulations of individual dVRB neurons were so varied that no discernable pattern could be found. Again, the responses to the three whine calls were neither distinct from the other call types nor from each other.

Figures 6-7 and 6-8 show the RMI values for each call type in each neuron. Figure 6-7 shows the 204 neurons that gave only enhanced responses. The neurons in this group show a range of strengths of enhancement depending on call type, although with no apparent patterns.

30 dVRB neurons showed exclusively suppressed responses caused by electrical stimulation, shown in figure 6-8 A. This is greater than AI, even when taking the differences in sample number into account. 15 of these neurons only gave significantly modulated response to a single call type. The others showed a variety of response strengths depending on call type, though the range of RMI values within neurons of this group did not extend to that of the all positive group.

Figure 6-8 B shows the 4 dVRB neurons that gave both enhanced and suppressed responses, depending on call type. This is by far the smallest group and there is no pattern to the call type responses. The strengths of response modification in each neuron, however, are relatively symmetrical about the zero RMI level. It would be useful to acquire more experimental data to determine if this pattern represents a true feature of this class of neuron.

A further 32 neurons showed no significant changes in their response to any of the call types. Therefore, the 238 total dVRB neurons displaying some form of AV activity represents a higher proportion of the total auditory neurons in this region than was observed in AI.

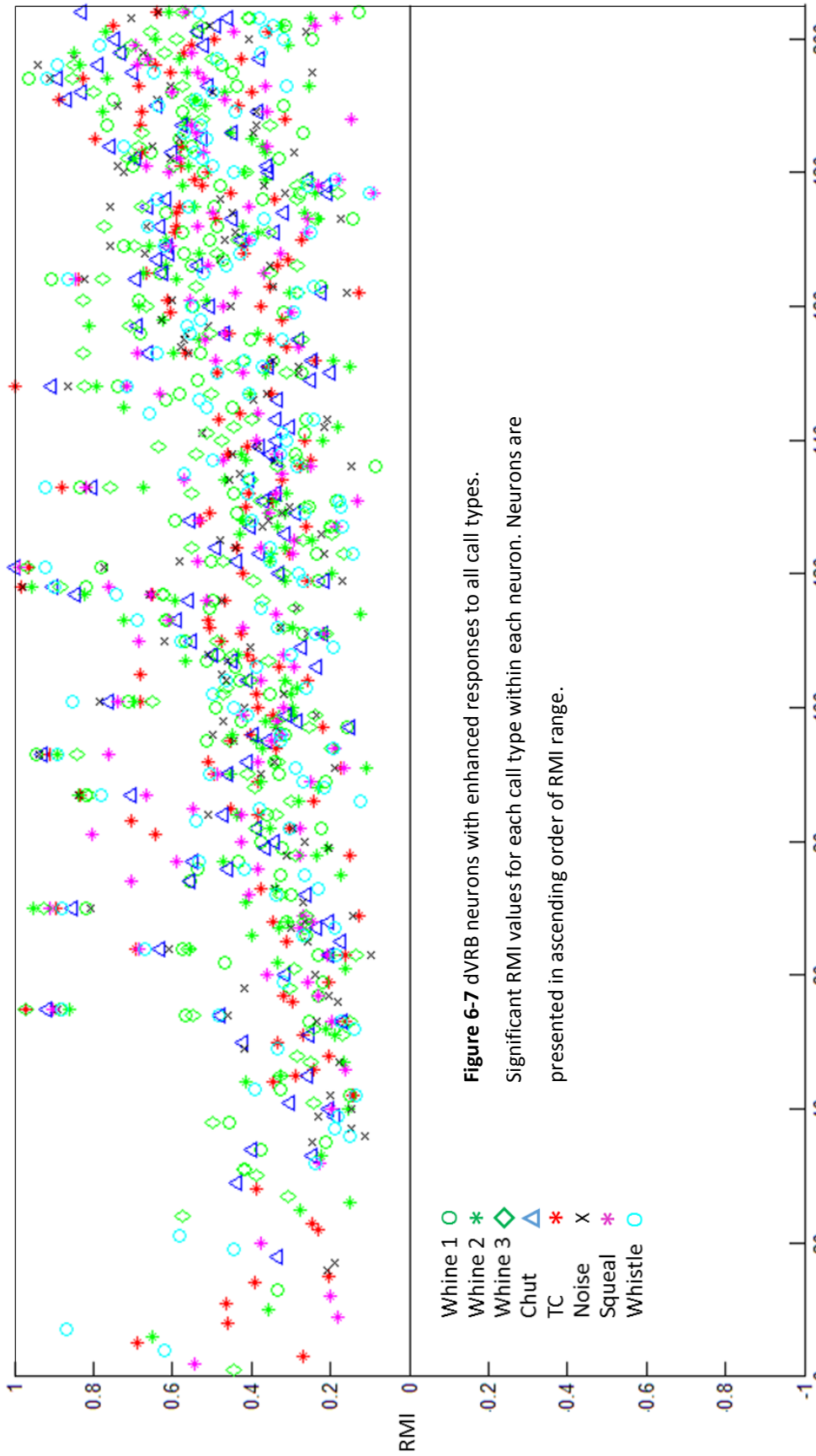



**Figure 6-7** dVRB neurons with enhanced responses to all call types.

Significant RMI values for each call type within each neuron. Neurons are presented in ascending order of RMI range.

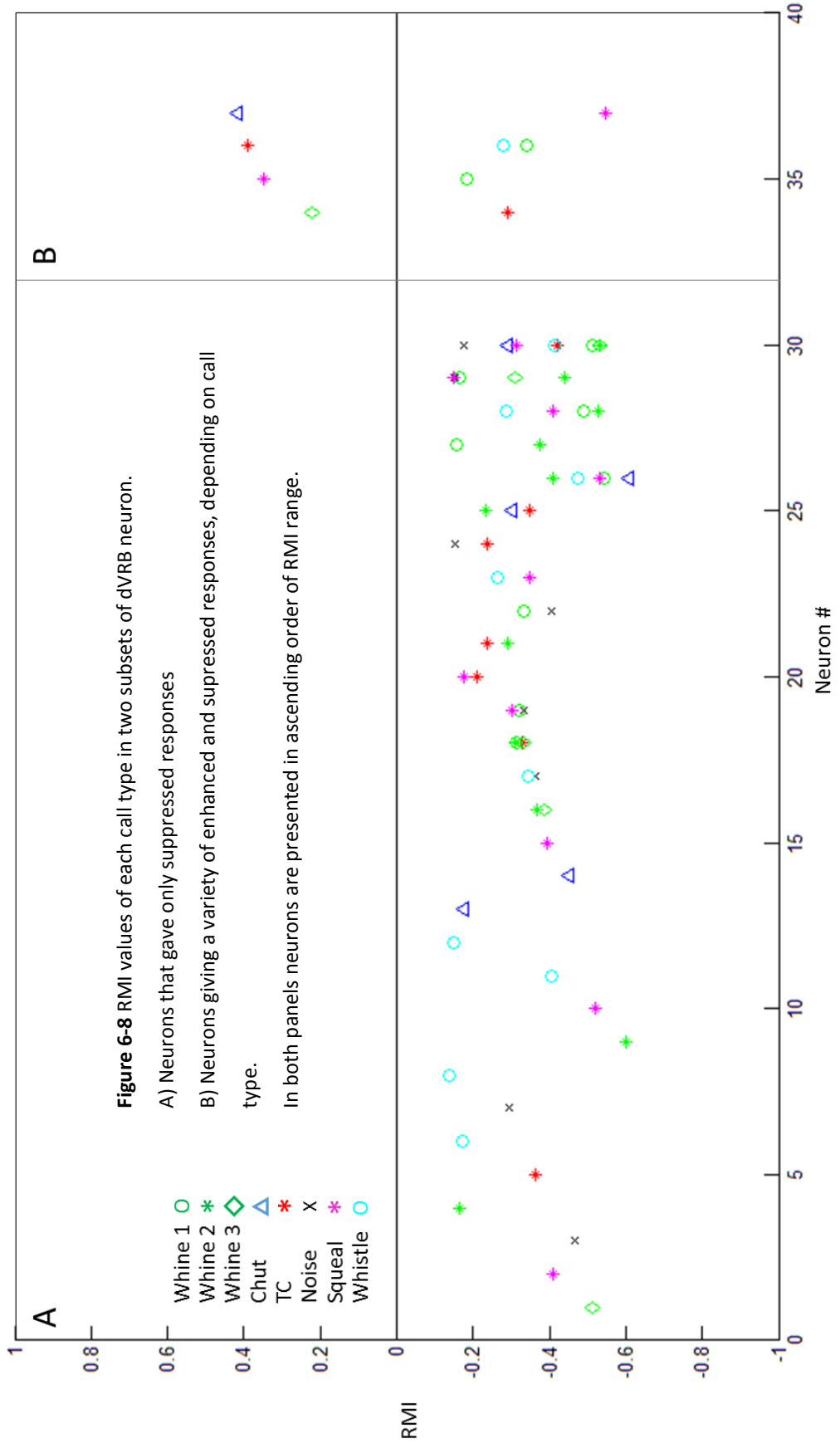



**Figure 6-8** RMI values of each call type in two subsets of dVRB neuron.

A) Neurons that gave only suppressed responses

B) Neurons giving a variety of enhanced and supressed responses, depending on call type.

In both panels neurons are presented in ascending order of RMI range.

**6.5 The effects of electrical stimulation of BA in dVRB**

The histograms in figures 6-9 and 6-10 show the effect of electrical stimulation alone, calculated in the same way as described for AI (see section 6.3). Figure 6-9 shows the initial 10-100 ms period, prior to auditory presentation.

137 dVRB neurons showed significant changes in firing rate caused by BA stimulation. The majority of these (123 neurons) were enhanced, with the strengths of these effects covering a broad range, as was the case in AI. Only 14 neurons were suppressed during this time period, these also displayed a broad range of effect strength. In AI, there were no suppressed neurons. The results from dVRB suggest that the smaller number of recorded neurons in AI may be the reason that suppression was not observed.

Modulation of firing rate in advance of a self-produced vocalisation has been reported in marmoset auditory parabelt during spontaneous vocalisation. As in AI this could be up to 400 ms prior to vocal onset, with no significant difference between the two areas. Differences in latency were observed within neurons in their response to different call types (Eliades and Wang, 2013). In squirrel monkey, short (5 ms) stimulation of ACC or dorsolateral prefrontal cortex (dlPC) produced modifications of firing rate of STG neurons in the period immediately following stimulation (Alexander et al., 1976; Müller-Preuss et al., 1980). Interestingly, whilst ACC stimulation produced predominantly suppressive effects, dlPC stimulation gave an even distribution of suppression and enhancement. This suggests that the results of the present study are likely to reflect the difference in activation of the vocal production system rather than interspecies variation; an assertion that could be readily tested using the same protocol.

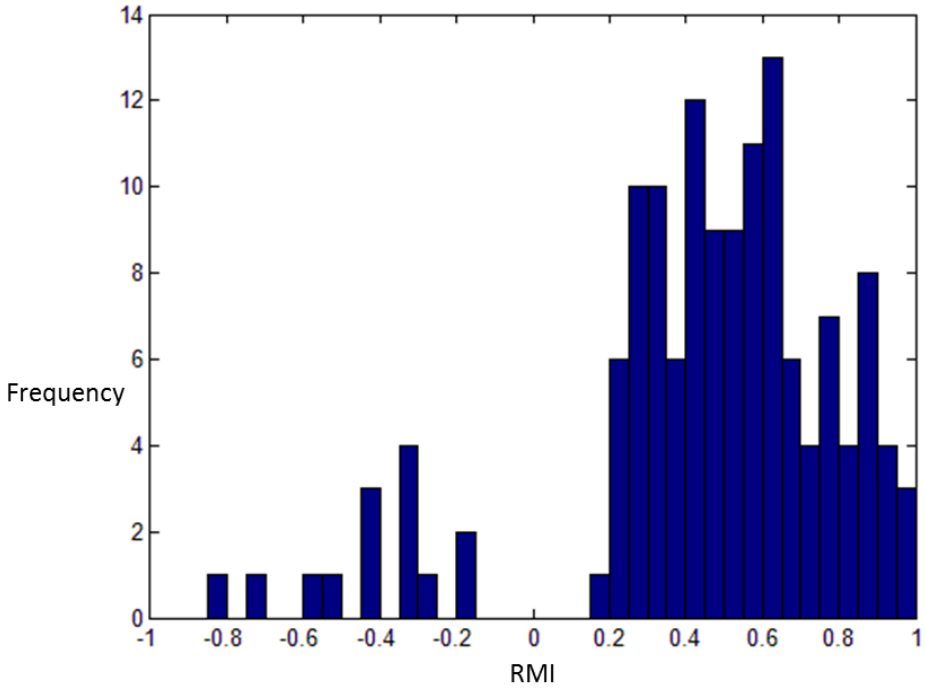



**Figure 6-9** Electrical stimulation of BA causes changes in spontaneous spiking activity in 137 dVRB neurons. Histogram of RMI values calculated from pooled spike counts in the period 10-100 ms. Bin size 0.05 RMI.

Figure 6-10 reports the persistent effect of stimulation in the period 850-1000 ms, that is, 50 ms after the cessation of the electrical stimulation. In this time period the effects are more evenly distributed between suppression and enhancement. Some 48 neurons showed significant changes in firing rate as a persistent effect of BA stimulation. Of these, 29 were enhanced and 19 were suppressed. Again, the disparity between the sample sizes for AI and dVRB may be the reason that only 2 AI neurons showed significant firing rate changes in this time period.

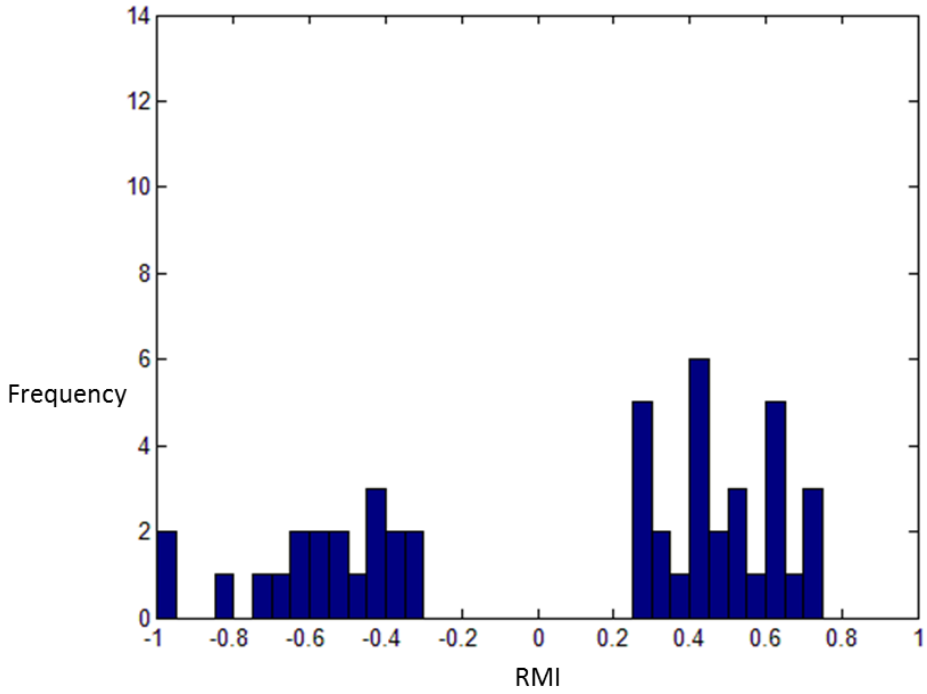



**Figure 6-10** Electrical stimulation of BA has an effect on spiking activity that persists for up to 150 ms after its cessation. Histogram of RMI values for 48 significantly modulated dVRB neurons, calculated from pooled spike counts of the chut and whistle trials in the period 850 - 1000 ms. Bin size 0.05 RMI.

### 6.6 Summary

Within each cortical area, the distributions of response modulations are remarkably similar for all call types. Between areas, dVRB showed higher overall enhancement of call response that AI. It is interesting that, whilst the population effects of BA stimulation are remarkably uniform, the response modulations within individual neurons of both cortical areas were varied.

In neurons of both cortical areas, the effects during BA stimulation alone show a similar pattern: a high proportion of neurons show significant modulation of firing rate. Of these, all (AI) or most (dVRB) show enhanced firing rates with a broad distribution of effect strengths.

The persistent effects of BA stimulation affected proportionally far fewer neurons in both cortical regions. In dVRB these modulated neurons were relatively evenly distributed between suppression and enhancement. Further experimental data would be required before the distribution of effects in AI could be determined.

# Discussion

## 7.1 Eliciting vocalisations using microstimulation

### 7.1.1 Comparison between electrically elicited and spontaneous vocalisations

As detailed in the introduction, a limited number of studies have elicited vocalisations from either awake or anaesthetised guinea pigs using electrical or chemical stimulation. In each case these calls were reported as natural sounding (Kyuhou and Gemba, 1999, 1998; Martin, 1976; Sugiyama et al., 2010). Only one of these studies showed spectrographs comparing two electrically elicited calls with their spontaneously produced counterparts, one of which I assert is named erroneously.

The guinea pig literature is not unusual in this regard. It has long been a central tenet of all vocal production research, that stimulation of PAG and all areas upstream of it yield natural sounding vocalisations (Gruber-Dujardin, 2010; Jürgens, 2009). The extent to which electrically elicited vocalisations can be considered natural is addressed here for the first time by making quantitative comparisons.

For six of the eight vocalisations described in the present study, the accepted principle of vocal production holds true. The only exception is the chutter. Both types of electrically elicited chutter share similar ranges in all parameters except for the $F_0$ gradient with their spontaneous counterpart. Initially, the flat chutter was named purr due to its regular pulse period and flat gradient. Upon comparison to the spontaneous purr, however, it was found to occupy a higher frequency domain. As the vocal apparatus of a guinea pig grows, the fundamental frequency of its purr drops. The flat chutter could potentially be the purr of a small guinea pig infant, but by 100 days post partum purrs are emitted with $F_0$ of ~250 Hz (Grimsley et al., 2011).

In light of the analyses performed in this study, it appears that Kyuhou and Gemba (1998) misnamed one of their electrically elicited calls. Figure 7-1 is adapted from their figure showing comparison between a spontaneous purr and, what they claim to be, an electrically elicited purr. Though the precise spectral properties cannot be assessed from the figure, it is clear that their electrically elicited call occupies a higher $F_0$ range and has a consistent positive gradient. Making it a likely match for the rising chutter described as defined in section 3.1.6. The authors compared their erroneous result with a cat-vocalisation study based on its social/emotional meaning, when purr and chutter have contrasting meanings (Zhang et al., 1994).

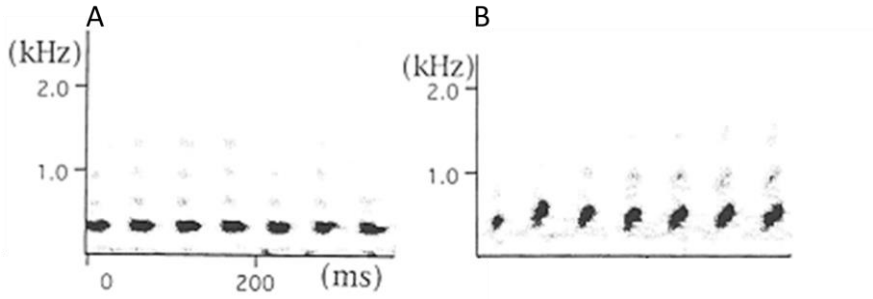



**Figure 7-1** Spectrograms of spontaneous purr (A) and an electrically elicited call (B) purported to be purr. Adapted from Kyuhou & Gemba (1998).

If this mistake could be made when using spectrograms to compare call types, it is likely that similar mistakes could easily be made if researchers were dependent on listening abilities alone. This finding raises important questions about the physiological processes involved in the spontaneous chutter because, to date, the vocal loci in PAG were thought to function independently of each other. In the present study, these two subclasses of call had representations in PAG at adjacent loci, separated by 200 µm. Such a finding was not unique to chutter as, on several occasions, a difference of 200 µm yielded a different call. No conclusions can be

drawn from this fact alone. Rather, this anatomical location would serve as a starting point for future anatomical and physiological study. Do these loci interact with each other in PAG? Or further downstream at the level of premotor neurons?

There are a number of interesting observations regarding the other calls:

The scream/squeal call series had a regular pulse period and a smooth and gradual progression in spectrotemporal structure. Though this would be unnatural in an adult guinea pig, these squeal series (but not the screams) resemble the isolation call produced by pups when they are left alone (Kyuhou and Gemba, 1998; Monticelli et al., 2004). So these regular rhythmic squeal series could be considered natural, only that they would never be produced by an adult. Adults, however, do produce screams in series (Berryman, 1976). Unfortunately, no such series were recorded by Grimsley (Grimsley, 2008), so it is impossible to say how they compare with the electrically elicited calls.

The chut call is interesting because both the spontaneous and electrically elicited calls share an irregular pulse period. This is the first time that chut has been reported using microstimulation. This unexpected similarity is distinct from all the other call types in this study. A review of primate vocal production literature has found no examples of electrically elicited calls with similar irregularity. In bat, however, complex motifs of communication calls have been elicited from the ACC (Schwartz, 2013). No purpose for chut's irregular pulse period has been proposed. The fact that this property is conserved in electrically stimulated calls warrants further investigation.

For the first time a toothchatter has been elicited by stimulation of guinea pig brain. Given its rarity in the current study it is unsurprising that it has not been reported by others. The range of pulse periods for the electrically elicited TC is greater than for the spontaneous calls, although the number of samples/animals from which this range was taken was not reported (Grimsley, 2008). It has been suggested that the rate of TC has some social significance during violent confrontation between GPs (Arvola, 1974). Although, upon reading Arvola's methods, it is hard to believe that accurate TC rate data was ascribed to the correct animal in a social encounter. Recently, wireless recordings of vocalisation-associated skull vibrations have been taken during social interactions (Hage and Jürgens, 2006b). A similar study in GP could determine if TC rates correlate with social behaviour, and whether the range of electrically elicited TC in the present study are within normal range.

### 7.1.2 Response to change in stimulation current

The only calls to change their spectrotemporal structure in response to changes in stimulation current were scream/squeal and whine. These aversive calls became higher frequency and more intense in response to increased stimulation current. As mentioned in 1.6 and 1.7, evidence from squirrel monkey research suggests stimulation of the amygdala and hypothalamus first produces a change in motivational state which is then followed by the corresponding vocalisation (Jürgens, 1976). A more recent study from the same institute correlated the level of aversive behaviour with the variation in spectral properties within aversive call types (Fichtel et al., 2001). Higher levels of electrical stimulation led to increased aversive behaviour which also positively correlated with calls of higher frequency, higher intensity and containing more noise. The prosodic properties of human speech share

a similar correlation with intensity of emotion (Hammerschmidt and Jürgens, 2007; Protopapas and Lieberman, 1997; Ruiz et al., 1996), as do the nonverbal vocalisations of babies (Rothgänger, 2003). In the present study it can be assumed that higher stimulation current corresponds with the activation of a greater number of neurons, i.e. activating a greater volume of brain tissue (Rowley et al., 1996). Were the guinea pig awake, this would correspond with a more strongly aversive motivational state, as is indicated by the spectrotemporal response to current change. This property of the vocal production system is later discussed in the context of audio-vocal interactions (see section 7.3.3).

### 7.1.3 Brain areas from which the vocalisations were elicited

Primate research has reported that, with the exception of PAG, loci relating to specific call types are not confined to discrete subnuclei (Jürgens, 2009; Martin, 1976; Robinson, 1967). My histological mapping data in GP agrees with this. Previous attempts to map GP vocal production loci suffer from the lack of spectral analysis, so no comparisons can be made with my data (Martin, 1976).

### 7.1.4 Amygdalar vocal production regions

This study is the first to produce vocalisations by stimulating the guinea pig amygdala. Furthermore, it is also the first nonprimate species in which activation of this nucleus has yielded two distinct call types. As previously mentioned (see section 1.7), two possible descending amygdalar pathways were shown independently in two different nonprimate species. Evidence for the existence of both pathways in a single species, squirrel monkey, was later provided (Jürgens, 1982).

Here, the amygdalar whine and TC loci were always spatially distinct. These call types also represent contrasting motivational states in awake animals (Berryman, 1976). These facts suggest that the same two descending amygdalar pathways are present and functionally active in the guinea pig. This strengthens the case for using guinea pig as an experimental model with which to address some of the outstanding questions in this area.

### 7.1.5 Why was the vocal repertoire incomplete?

Of the 11 vocalisations reported in awake, spontaneously vocalising guinea pigs 7 were produced during the present study. There are several possible reasons for this incomplete repertoire:

This study was not exhaustive, neither in terms of the anatomical areas nor the parameters of microstimulation. All the stimulated nuclei were those which, between them, according to squirrel monkey research, should have yielded every vocalisation (Jürgens, 2009). It cannot, however, be said that each of these nuclei were comprehensively sampled with electrode tracks (see figure 3-37). As previously stated, modification of the frequency of electrical stimulation cannot change the pulse rate of elicited calls. It has, however, been shown that changes in frequency at certain loci can change the call type that is produced. The frequency ranges for the different calls were discrete and it was presumed that different classes of neuron were being activated (Jürgens, 2002). Similar stimulation methods in the anaesthetised GP may reveal the remaining call types.

It was observed that an increase in anaesthesia level could suppress a given call at a given locus. So it is possible that it could completely suppress the production of

certain call types, even at the lightest level of anaesthesia that was possible using the current protocol. Furthermore, anaesthesia level may affect the observed distribution of call production loci, i.e. some of the call types in the current study may have additional loci that would only be apparent in an unanaesthetised animal. The disparity between experiments, in terms of call types that were recorded, could be indicative of this effect. Further work using chronically implanted electrodes would be needed to confirm this hypothesis.

### 7.1.6 Chemical stimulation

Chemical stimulation has been used to elicit vocalisations and provide information as to the types of neuron being activated. Cholinergic agonists can elicit vocalisations from the amygdala and hypothalamus, but not in the PAG or ACC. The opposite is true for glutamatergic stimulation (Brudzynski and Bihari, 1990; Jürgens and Richter, 1986; Lu and Jürgens, 1993; Manteuffel et al., 2007).

Comparative studies in squirrel monkey used electrical stimulation to identify vocal production loci, and then stimulated these loci chemically. In brain areas corresponding with those stimulated in the present study, each chemical compound was able to elicit vocalisation at only a small percentage of the loci (Jürgens and Richter, 1986; Lu and Jürgens, 1993). There were no instances of a given locus producing different call types depending on whether electrical or chemical stimulation was used. One study in cats, however, found several loci in the hypothalamus and amygdala that would produce vocalisation only when stimulated chemically (Baxter, 1967).

The limited amount of research using chemical stimulation has yielded some interesting results. Future work could use chemical agonists to a wider range of cell types, along with combinations of such chemicals, to elucidate the neural circuitry of the various vocal production areas.

### 7.1.7 Habituation

The during-stimulation calls from areas upstream of PAG show habituation as has been described by previous researchers. From current understanding of habituation, the timescale over which this occurs and then recovers fits with a 'deletion model' (Zucker and Regehr, 2002), wherein high levels of activity deplete the reserves of neurotransmitter vesicles within the neurons. These are then replenished over the course of several minutes. These different habituation effects presumably reflect the type/s of neuron that are responsible for vocalisation. Chemical stimulation has highlighted differences in neuron type between the vocal production nuclei. These findings are a useful guide to anyone wishing to investigate further, but they do not provide conclusive answers. For example, glutamate can elicit vocalisations from PAG and ACC yet they respond differently to repeated electrical stimulation.

### 7.1.8 Enhancement

Habituation was not observed for the post-stimulus scream/squeal series, regardless of the brain area from which they were elicited. On the contrary, repeated stimulation caused calls to increase in intensity and duration. This indicates that numerous brain regions are involved in the production of these calls, so that any single area does not become depleted of release-ready neurotransmitter.

This enhancement effect has not been reported previously yet it is consistent with the current literature. In awake primates post-stimulus call series lasting up to several minutes can be elicited (Ploog, 1981; Robinson, 1967). Enhancement in the anaesthetised GP, with call series increasing in duration up to 30 seconds, probably reflects generalised arousal caused by the stimulation counteracting the anaesthetic (Angel, 1993; Kapp et al., 1994).

### 7.1.9 Dual stimulation

The original dual stimulation hypothesis, as stated in section 4, was that concurrent stimulation of two post-stimulus scream/squeal loci would have a summative effect. At the time it was not clear how widespread these loci were. The hypothesis was not disproven, yet the effect was too diffuse to allow for any specific anatomical or physiological inferences. Reciprocal loops between neocortex, thalamus and the nuclei of the basal ganglia have an involvement in mediating emotion, motor control, learning and cognitive abilities (Middleton and Strick, 2000; Parent and Hazrati, 1995). A resounding feature, however, is that reciprocal connections have very high anatomical precision. Therefore, the current literature was unable to shed any light on the pervasive effect observed.

The second test was unplanned yet provided an unexpected benefit. Dual stimulation of during-stimulus and post-stimulus call loci, provides evidence in support of Robinson's claim that during- and post-stimulus call types are controlled by separate neural circuitry (Robinson, 1967). Stimulation of an amygdalar whine locus also caused enhancement of a post-stimulus scream/squeal series in a similar manner to the previous dual stimulation experiment. Repeated stimulation led to habituation of the whine whilst the scream/squeal series was enhanced. This result is consistent

with both a localised depletion and an excitatory re-entrant system, for whine and scream/squeal, respectively.

The alternative hypothesis was that these two, simultaneous effects are a result of the same stimulation-related short-term plasticity. Elicited vocalisations always fell into distinct categories (as defined in section 3), even when loci were separated by 200 μm. This finding suggests that the neural circuits responsible for a given call type are capable of suppressing those of other call types.

The results presented in figure 4-9 (section 4.2) were ambiguous; the two time course progressions were neither similar, nor significantly different. This hypothesis is worthy of further investigation, but alternative experimental methods would be required in order to provide sufficient evidence. The cellular basis of the vocal production system is a new area of investigation, and there is very little literature on the subject. It may be necessary to use neuron-type specific methods to elicit vocal production, such as those described in section 7.1.6, or to use electrophysiological recording techniques.

There were only three instances of dual stimulation at two during-stimulus call loci. Of these, only the single unilateral test, amygdalar whine with hypothalamic chut, resulted in altered vocal output. The suppressive effect that hypothalamic activation had on the amygdalar vocalisation indicates that future dual stimulation work of this nature would provide useful information about their interactions. A specific, hypothesis-driven study could determine whether this suppression occurs in the hypothalamus or further downstream in the PAG. The confounding issue of habituation could be avoided by allowing several minutes between each repeat. The use of either chemical or physical lesions would also be a useful and feasible addition.

### 7.1.10 Conclusions: Vocal production system

The analyses performed in the current study found only one call type that was appreciably different from its spontaneously produced counterpart. For the reasons described above it is easy to understand why this has gone unnoticed. Indeed, the flat chutter was initially assumed to be purr. Only the quantitative analysis revealed the non-overlapping frequency ranges, compelling its reclassification.

The results for this vocalisation cast some doubt over a central assumption of vocal production research. The effects on chutter, however, are not generalised to the other guinea pig calls. So it would be remiss to extrapolate to other species. This finding highlights the necessity to perform similar analyses in all future vocal production research (taking account of age/size of the animal), and to take caution when interpreting the existing literature. By contrast, the abnormal calls reported from stimulation of the pontine reticular formation in squirrel monkey (i.e. downstream of PAG) leave no possibility of confusion with the normal repertoire (Jürgens and Richter, 1986).

Vocal production anatomy and response to electrical microstimulation is consistent across a range of species including primates, rodents, bats and cat. I have shown that guinea pig is also a suitable model with which to study this conserved system. Furthermore, the discovery of multiple call types from amygdala stimulation strengthens the model with respect to its similarity to a primate species.

## 7.2 Deep ventrorostral belt (dVRB)

A new auditory sensitive region of guinea pig neocortex has been discovered. Named deep ventrorostral belt (dVRB), it is especially selective for conspecific vocalisations

and is involved in AV interactions. These findings indicate that auditory processing of complex sounds in GP may be similar to the ventral processing pathway described in primates (Rauschecker and Tian, 2004). In primate, the definitive properties of auditory cortical belt regions are their thalamic connections (Hackett et al., 1998). In GP, thalamic connections to AI match those seen in primate (Wallace et al., 2000). Similar histological testing in VRB and dVRB would be needed before further comparisons between primates and rodents could be made.

### 7.3 The basal amygdala plays a role in the audio-vocal system

Previous audio-vocal research has focused on either motor planning brain areas, such as ACC and prefrontal cortex, or motor control areas, such as PAG and the pontine reticular formation (see section 1.14). To date, the involvement of emotion-mediating brain areas has not been investigated. In addition to its role in vocal production, the amygdala is also involved in vocal perception. The fear response in Pavlovian-conditioned rats is abolished following destruction of the basal amygdalae, and humans show impaired auditory recognition of fear and anger following bilateral amygdala lesions (Maren et al., 1996; Scott et al., 1997). I have shown that activation of the basal amygdala can modify the sensory cortical representation of auditory stimuli.

The physiological responses at auditory cortex demonstrate a complex interaction between amygdala and auditory stimulation, in a manner consistent with the efference copy model of sensorimotor interactions. This work does not contradict any of the current literature. It is likely that numerous brain regions act in concert during spontaneous vocalising. Rather, I assert that the basal amygdala ought to be added to that body of knowledge.

### 7.3.1 Anatomical correlates of the amygdalar corollary discharge

By what pathway or pathways might the amygdala be having its effect? As mentioned in section 1.23, direct pathways exist from BA to AC, MGB and IC. There are, however, several indirect pathways by which the amygdala could have its effect. None of which are mutually exclusive; multiple efference copies may be sent via different pathways.

Any part of the descending vocal production system could be involved in the generation of efference copies. Several of which have proven audio-vocal functions (see section 1.1.4). The amygdala has reciprocal connections with ACC which, also, is known to have an audio-vocal role (Morris et al., 1998; Müller-Preuss et al., 1980).

Neural tract tracing would be an obvious next step to answering some of these questions. It could also prove useful to move the stimulating electrode along the descending whine pathway. Would stimulation of a whine production locus in the hypothalamus or PAG have similar effects? Moving the recording site could also be informative. For example, how would inferior colliculus or other nuclei of the ascending auditory respond to these test conditions?

### 7.3.2 Physiological responses at auditory cortex

Attempts to understand the neural coding of complex sounds such as conspecific vocalisations and speech have only recently begun. Cortical responses are abstract and cannot be predicted from CF (Grimsley et al., 2012; Perrodin et al., 2011; Wang et al., 1995). Considering AV interactions adds a further layer of complexity. In the present study, this is one reason why the varied response modulations within individual neurons defied classification. It is interesting that the populations of

neurons within each cortical area showed similar response modulations for all call types. As yet there is no clear explanation for this finding. In future, a more sophisticated analysis based on information theory may provide useful insight.

Stimulation of different vocal production loci causes vastly different effects in the same auditory cortical area in the same species (Alexander et al., 1976; Müller-Preuss et al., 1980). It was, perhaps, to be expected that the effects of BA stimulation were different from both these previous reports. The protocol used here could be easily adapted to allow comparison between the effects of stimulation in these different loci. Further to this, by incorporating dual stimulation, it would be possible to investigate the effects of different stimulation loci in the same neuron. It would be interesting to know whether AV projections converge in AC to effect the same neurons.

### 7.3.3 Frequency-specific aspects of AV interactions

I and others have shown that increased stimulation current in BA gives vocalisations with increased the $F_0$ (Jürgens, 2009). And, in awake, freely behaving animals, this increased current also leads to increased aversive behaviour (Fichtel et al., 2001). If, as I have suggested, BA is involved in audio-vocal interactions, then changing the stimulation current ought to produce changes in EC that correspond with the expected change in auditory input. This hypothesis could be easily tested using the existing experimental protocol.

Of particular interest to the present study are the autistic spectrum disorders (ASD). Individuals with ASD often have difficulty with language acquisition and prosody production (McCann and Peppe, 2003; Rapin and Dunn, 2003). Russo et al. showed a

reduced ability to compensate their vocal output when presented with real-time pitch-shifted auditory feedback (Russo et al., 2008). In tandem with these findings, several researchers have suggested amygdala malfunction as a cause of many ASD symptoms (Baron-Cohen et al., 2000; Schultz, 2005). As yet, no one has investigated this correlation. These facts, combined with the data presented here, suggest that such a study would be useful.

### 7.3.4 Timing of AV interactions

Vocalisation-related changes in auditory cortical firing has been observed up to 400 ms in advance of vocal onset in marmosets (Eliades and Wang, 2003). In the present study, the latency of vocal onset following electrical stimulation is approximately 400 ms, and modified firing in some AI and dVRB neurons was seen in the period 10 – 100 ms. These suggest that, in agreement with the marmoset study, the corollary discharge reaches the cortex in a similar timeframe.

An audio-vocal system needs to sustain its activity for a length of time sufficient to cover the latency in vocal production and the duration of the vocalisation itself. From the data presented in section 3 this could be in the region of 1 – 2 seconds. This could require temporal coordination within a population of neurons. Previous audio-vocal studies using microstimulation were unable to remove the electrical artefact from the neural recordings, so were limited to observing effects in the time period immediately following its cessation (Alexander et al., 1976; Müller-Preuss et al., 1980). I was able to eliminate the stimulation artefact (see section 2.5.2), revealing differences between the during-stimulation and post-stimulation periods that were similar for both AI and dVRB.

Defects in synchronisation of audio-vocal interactions in human are the cause of stuttering. This condition can be ameliorated using delayed auditory feedback, and non-stutterers develop an artificial stutter when their feedback is manipulated in a similar way (Howell et al., 2000; Timmons and Boudreau, 1972). The experimental protocol I have developed provides a tractable model to investigate the effects of audio-vocal synchronisation.

### 7.3.5 Conclusions: Audio-vocal interactions

It is well established that the emotional system has a role in mammalian vocal production and human speech prosody; an audio-vocal system would be insufficient were it not to take these into account. For the first time, the potential for these interactions has been considered, and a neurobiological correlate was demonstrated.

The guinea pig was shown to be a useful model for the study of audio-vocal interactions, and the experimental protocol that was developed has the potential to provide more informative data in the future. The discovery of dVRB has broadened the scope for future guinea pig auditory physiology. To date, only primates were thought to have auditory parabelt regions. Other nonprimates, especially vocal learners such as bats and mice, may have equivalent cortical regions that would be worthy of investigation.

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