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REVIEW

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Probing forebrain to hindbrain circuit functions in *Xenopus*

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Abstract

The vertebrate hindbrain includes neural circuits that govern essential functions including breathing, blood pressure and heart rate. Hindbrain circuits also participate in generating rhythmic motor patterns for vocalization. In most tetrapods, sound production is powered by expiration and the circuitry underlying vocalization and respiration must be linked. Perception and arousal are also linked; acoustic features of social communication sounds—for example, a baby's cry—can drive autonomic responses. The close links between autonomic functions that are essential for life and vocal expression have been a major *in vivo* experimental challenge. *Xenopus* provides an opportunity to address this challenge using an *ex vivo* preparation: an isolated brain that generates vocal and breathing patterns. The isolated brain allows identification and manipulation of hindbrain vocal circuits as well as their activation by forebrain circuits that receive sensory input, initiate motor patterns and control arousal. Advances in imaging technologies, coupled to the production of *Xenopus* lines expressing genetically encoded calcium sensors, provide powerful tools for imaging neuronal patterns in the entire fictively behaving brain, a goal of the BRAIN Initiative. Comparisons of neural circuit activity across species (comparative neuromics) with distinctive vocal patterns can identify conserved features, and thereby reveal essential functional components.

KEYWORDS

fictive respiration, vocalization, pattern generation

1 | INTRODUCTION

Xenopus has been a highly productive model system for developmental and cell biology (Gurdon & Hopwood, 2012; Wühr et al., 2015). Genomic resources (Pearl, Grainger, Guille, & Horb, 2012) are supporting ongoing efforts in gene regulation and evolution (Furman et al., 2015; Session et al., 2016). Analyses of neural circuits using the isolated *Xenopus* nervous system (reviewed here), together with emerging technologies for whole brain imaging (Tomer et al., 2015), will significantly advance understanding of how these circuits generate motor patterns. The *ex vivo* brain generates patterns of nerve activity that match the sound patterns of vocal communication signals (calls) and respiration (Rhodes, Heather, & Yamaguchi, 2007; Zornik & Kelley, 2008). This fictively behaving preparation provides substantive insights not only into the function of experimentally challenging hindbrain circuits, but also into how those circuits are accessed and coordinated by descending input from forebrain regions that decode social cues (Hall, Ballagh, & Kelley, 2013). Hormonally-regulated, sexually differentiated circuit features are maintained *ex vivo*, facilitating analyses of mechanisms underlying endocrine action (Zornik & Kelley, 2011; Zornik & Yamaguchi, 2011). The *ex vivo* fictive preparation is accessible across species (Leininger & Kelley, 2013; Leininger, Kitayama, & Kelley, 2015) providing insight into how neural circuits are tuned to generate phylogenetic diversity in vocal patterns. Retuning in response to hormones can also be followed across species with divergent modes of primary sex determination (Furman & Evans, 2016; Mawaribuchi et al., 2016; Roco et al., 2015). This approach (comparative neuromics) provides an important advantage for whole brain imaging studies: the ability to identify conserved and variable features across species in order to tease apart which aspects of a circuit's activity are essential for its function (Gjorgjieva, Drion, & Marder, 2016; Katz, 2016).

2 | VOCAL PRODUCTION; HINDBRAIN CIRCUITRY

In *Xenopus*, sound pulses are generated by contraction of paired laryngeal muscles in response to activity on the vocal nerve (Tobias & Kelley, 1987; Yamaguchi & Kelley, 2000). This nerve arises from

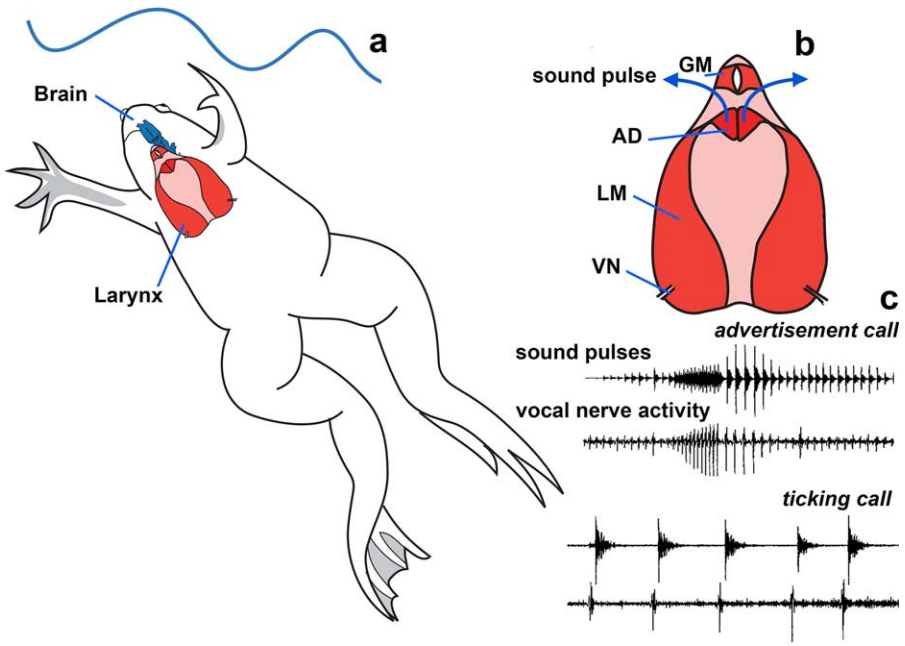

FIGURE 1 Vocal production in *Xenopus*. (**a**) *Xenopus* call while submerged. (**b**) Air travels through the glottis to the lungs. The glottis is closed during vocalization. Each sound pulse is generated by separation of paired arytenoids disks (AD), in response to contraction of laryngeal muscles (LM) driven by activity on the vocal nerve (VN). **(c)** Nerve activity patterns (lower traces) match the sexually differentiated patterns of sound pulses (upper traces) in the male *advertisement call* and the sexually unreceptive female *ticking call*

motor neurons located in nucleus ambiguus (NA) of the hindbrain and includes laryngeal motor neuron and glottal motor neuron axons as well as axons that innervate the heart (Simpson, Tobias, & Kelley, 1986). The glottis gates airflow between the mouth and the lungs through the larynx (respiration) and is closed during sound pulse production (Yager, 1992; Zornik & Kelley, 2008). Thus, in *Xenopus*, as in most vocal vertebrates (Schmidt & Goller, 2016), sound production is linked to respiration even though not powered by expiration.

The pattern of vocal nerve activity recorded *in vivo* matches the pattern of sound pulse production of actual male and female calls (Figure 1c). These patterns can also be recorded *ex vivo* (Figure 2a) from the vocal nerve in an isolated brain and are thus termed *fictive calling* (Figure 2b,c). When the larynx and the brain are isolated together (Figure 2d), glottal muscle activity patterns that accompany contractions can be recorded from glottal motor neurons (Figure 1e); the corresponding nerve activity patterns are termed *fictive breathing*. The *ex vivo* preparation in *Xenopus* thus provides an exceptional opportunity to study the linkages between neural circuit elements for vocalization and respiration.

In *X. laevis*, the *ex vivo* preparation has revealed key features of hindbrain circuitry as well as the connections between forebrain and hindbrain nuclei that initiate vocalizations and control vocal patterning (Brahic & Kelley, 2003; Hall et al., 2013; Figure 3a,b). The *fictively calling* brain has facilitated the analysis of the role of two groups of hindbrain neurons linked to respiration in many other vertebrates. One is a nucleus, DTAM, in the rostral hindbrain that projects throughout NA (Figure 3b). DTAM is rhythmically active during *fictive advertisement calling* (Zornik, Katzen, Rhodes, & Yamaguchi, 2010; Figure 3c). The male-specific *advertisement call* (Figure 1) consists of fast and slow trills; DTAM controls fast trill duration and thus overall call duration and period (Zornik & Yamaguchi, 2012). The other respiratory-linked nucleus is NA in the caudal hindbrain that includes both glottal and laryngeal motor neurons. Nucleus ambiguus is responsible for the slow trill pattern initiated by descending input from DTAM (Figure 3c). Each half of the hindbrain includes independent vocal pattern generators and these are coordinated by an anterior commissure (AC) at the level of DTAM and a posterior commissure (PC) at the level of NA as well as by reciprocal ipsilateral and contralateral connections between DTAM and NA (Figure 3c). Activity in DTAM influences both vocalization and respiration. DTAM provides monosynaptic excitatory (glutamate) input to laryngeal motor neurons and excites inhibitory (GABA) interneurons that suppress activity of glottal motor neurons (Zornik & Kelley, 2008). Since *Xenopus* calls while submerged (Figure 1a), this inhibitory circuit element prevents water in the mouth cavity from entering the lungs via the larynx during vocal production.

3 | BREATHING AND CALLING: BRAIN CIRCUIT HOMOLOGS

Individual neurons within neural circuits can be identified by their molecular identities, for example, transcription factors, neurotransmitters, ion channels -as well as by their developmental origins and connections to other neurons. Molecular identities support homology (i.e. similarity across species due to inheritance from a recent ancestor) of the *Xenopus* caudal hindbrain nucleus

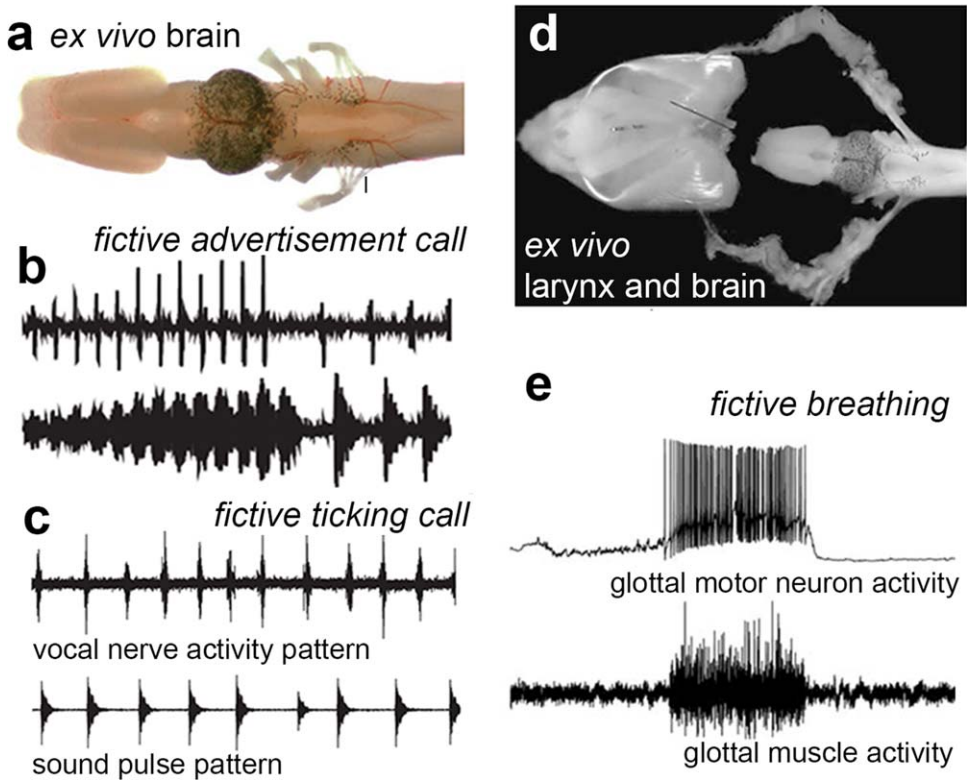

FIGURE 2 Fictive calling and breathing preparations in *X. laevis*. (**a**) The isolated (*ex* vivo) brain viewed from above (dorsally) from olfactory bulb rostrally (left) to the end of the hindbrain caudally (right). The most caudal rootlet of cranial nerve IX-X (indicated by the line) contains the axons of motor neurons that innervate glottal, laryngeal and heart muscles and comprises the vocal nerve. (**b**) Fictive *advertisement calling* (upper trace) can be recorded from the VN when the neuromodulator serotonin is bath applied to the *ex vivo* brain. The pattern of VN activity follows the pattern of a male *advertisement call* (lower trace). (**c**) Fictive *ticking* (upper trace) can be recorded from the VN when the neuromodulator serotonin is bath applied to the *ex vivo* brain. The pattern of VN activity follows the pattern of female *ticking* (lower trace). (from Rhodes et al., 2007). (**d**) The isolated (*ex* vivo) brain and innervated larynx viewed from above A branch of cranial nerve IX-X enters the larynx caudally and contains the axons of motor neurons that innervate glottal and laryngeal muscles. **e** Recordings from glottal muscles (lower panel; also see Figure 1**b**) reveal spontaneous bursts of activity that correspond to activity of hindbrain glottal motor neurons. This activity pattern produces the opening of the glottis that allows air to travel from the mouth through the larynx to the lungs and is thus termed *fictive breathing*

that includes laryngeal and glottal motor neurons (N.IX-X) to the NA of mammals and other vertebrates (Albersheim-Carter et al., 2016). As in other vertebrates, including mammals, neurons in *Xenopus* N.IX-X express a key hindbrain transcription factor, pHox2B, and originate from caudal hindbrain segments (rhombomeres). N. IX-X motor neurons are cholinergic and project to glottal, laryngeal or heart muscles.

Using these kinds of criteria, candidate homologs for nucleus DTAM in the rostral hindbrain include the paratrigeminal respiratory group (pTRG) in a non-vocal basal vertebrate, the lamprey (Missaghi, Le Gal, Gray, & Dubuc, 2016), and the parabrachial area (PBA) in vocal mammals (Dick, Bellingham, & Richter, 1994; Smith, Abdala, Borgmann, Rybak, & Paton, 2013). Neurons in the lamprey pTRG originate from rostral rhombomeres and provide glutamatergic innervation to NA. Neurons in the lamprey pTRG are rhythmically active and control expiration. Neurons in the mammalian PBA also express pHox2B, originate developmentally from rostral rhombomeres, provide glutamatergic innervation to nucleus ambiguus and are rhythmically active during respiration; PBA activity is also tied to vocalization (Hage et al., 2006).The role of DTAM in *Xenopus* vocalizations (shared with the PBA in mammals) could represent an exaptation (co-option) of an expiratory hindbrain circuit element present in basal vertebrates and preserved in many vertebrate lineages.

Another rhythmically active region, the preBötzinger nucleus, is located in the caudal hindbrain, ventral to NA, and appears essential for respiratory patterning in mammals including humans. Given the strong evolutionary conservation of the caudal rhombomeres from which this nucleus originates (Bass, Gilland, & Baker, 2008), homologs of the preBötzinger nucleus are likely contributors to respiratory and/or vocal patterns in other groups such as *Xenopus*. The pulmonary pattern generator of frogs, coextensive with NA, is a candidate homolog though circuit elements are not yet characterized molecularly (Smith et al., 2016). The *slow trill* pattern generator in *X. laevis* is contained within NA (Figure 3c) and could be homologous to the mammalian preBötzinger complex. Characterizing transcription factor expression and electrophysiological characteristics, including rhythmicity and connectivity, will address this question.

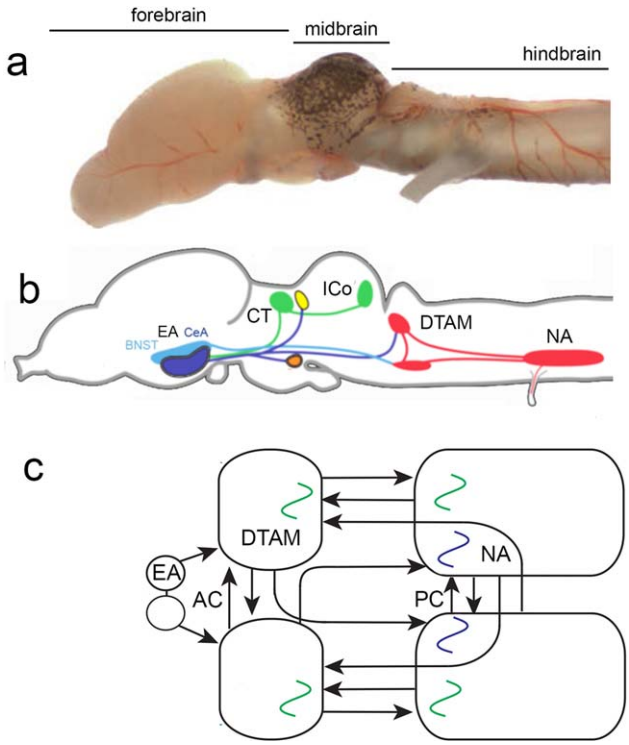

FIGURE 3 Initiation and production of vocal motor patterns in *X. laevis*. (**a**) The *ex vivo* brain (Figure 1**a**) now viewed from the side and illustrating subdivisions (hindbrain, midbrain, and forebrain) that include neural circuits participating in initiation of vocal patterns. In an adult male brain, nucleus ambiguus (NA) that includes glottal and laryngeal motor neurons (**b**) is ~1mm from rostral to caudal. (**b**) A current view of brain nuclei that participate in vocal patterning. The activity of neurons in the inferior colliculus (ICo) of the midbrain is preferentially driven by sound pulse rates characteristic of specific calls (Elliott et al., 2011). The ICo projects via the central nucleus of the thalamus (CT) to the central amygdala (Ce) in the ventral forebrain (Brahic & Kelley, 2003). Stimulation of either the CeA or the adjacent bed nucleus of the stria terminalis (BNST; together the extended amygdala or EA) *in vivo* initiates fictive advertisement calling (Hall et al., 2013). (**c**) A current view (from Yamaguchi et al., 2016) of the forebrain and hindbrain connections and activity responsible for initiating and coordinating *fictive calling*. This schematic, bilateral view (from the top) extends from the EA rostrally (left) to NA caudally. The hindbrain includes separate pattern generator circuits for the two components of the male advertisement call: fast trill and slow trill. The slow trill pattern generator (in blue) is co-extensive with NA while the fast trill generator (in green) is distributed between NA and a more rostral nucleus DTAM (used as a proper name). Coordination between the two halves of the hindbrain is accomplished by contralateral connections at the level of DTAM (the anterior commissure or AC) and NA (the posterior commissure or PC) and by bilateral connections between DTAM and NA

4 | VOCAL PRODUCTION; FOREBRAIN INITIATION

In *Xenopus laevis*, specific acoustic features of the calls of other frogs can evoke specific vocal response patterns. For example, female *ticking* transiently silences males (Elliott & Kelley, 2007) while intense male *advertisement calling* produces prolonged vocal suppression (Tobias et al., 2004). Selectivity for the specific temporal patterns of different calls can be demonstrated in a midbrain nucleus (Elliot & Kelley, 2011) and can be relayed to DTAM via a nucleus of the ventral forebrain (Figure 3b). Transcription factor expression and connectivity (Moreno & Gonzalez, 2007) support the homology of this forebrain nucleus to the central nucleus of the amygdala (CeA; Figure 4a,b), known to govern both autonomic responses and vocalizations in other vertebrates including mammals (Ma & Kanwal, 2014). Electrical stimulation in the *X. laevis ex vivo* brain reveals that the CeA and the adjacent bed nucleus of the stria terminalis (collectively the extended amygdala or EA) initiate *fictive advertisement calling* in male brains (Hall et al., 2013; Figure 4b–d). The output of the EA is inhibitory (GABAergic; Figure 4b). When EA output to DTAM is removed bilaterally and the anterior commissure is transected, the left and right brainstem produce uncoordinated *fictive fast trills* but *fictive slow trills* remain coordinated (Yamaguchi, Barnes, & Appleby, 2016; see Figure 3c). The *ex vivo* brain preparation of *Xenopus* can thus reveal not only detailed hindbrain neural circuit mechanisms but also potential roles for sensory input in initiating vocal responses.

5 | SEXUAL DIFFERENTIATION OF VOCAL CIRCUITS

The *Xenopus* vocal repertoire differs by sex (Figure 1). In *X. laevis*, as in most vertebrates, sexual differentiation reflects the secretion of steroids (androgens and estrogens) from the gonads during development or in adulthood (Zornik & Kelley, 2011). The male-typical *advertisement call* pattern can be evoked, even in adult females, by androgen treatment (Potter, Bose, & Yamaguchi, 2005). Some neurons in NA and DTAM express androgen receptor (a ligand-activated transcription factor) in both sexes (Perez et al., 1996). Following androgen treatment, *ex vivo* brains from adult females produce *fictive advertisement calling* rather than *fictive ticking* (Potter et al., 2011).The *fictively calling* adult female brain thus provides an opportunity to determine how neural circuits are retuned during the course of exposure to, and withdrawal of, androgens. In addition, the developmental effects of gonadal steroids on neural circuitry (the classical "organizational" effects (Wallen, 2009) experienced by males can be isolated from the effects of hormone secretions during adulthood (the classical "activational" effects) experienced both by males and androgen-treated females. Gonadal steroids regulate sensitivity to acoustic features of *Xenopus* vocalizations as well as motor patterns (Hall et al., 2016). Auditory sensitivity to species-specific sound frequencies in *advertisement calls* is greater in adult females due to ovarian hormones; steroids thus may also retune sensory circuits. As we understand more fully additional endocrine and sensory cues that govern behavioral interactions (Rhodes, Stevenson, & Ego, 2014), the *ex vivo* brain preparation should also prove valuable in uncovering responsible neural mechanisms.

6 | COMPARATIVE NEUROMICS; GENE EXPRESSION AND NEURAL CIRCUITS

Comparing neural circuit functions across species uses the long term "experiment" of evolution to uncover the genetic mechanisms

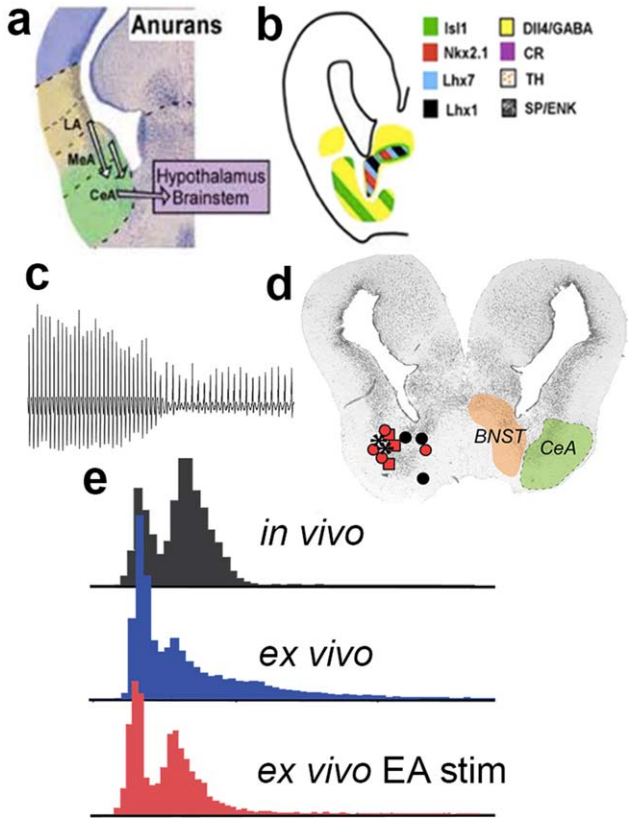

FIGURE 4 Molecular identity of forebrain nuclei that contribute to vocal initiation. (**a**) and (**b**) are modified from "Evolution of the amygdaloid complex in vertebrates, with special reference to the anamnio-amniotic transition," by Moreno and Gonzalez, 2007, Journal of Anatomy, 211, p 151. (c-e) are modified from "The Xenopus amygdala mediates socially appropriate vocal communication signals." by Hall et al., 2013, The Journal of Neuroscience, 33, p 14534. **(a)** The anuran amygdaloid complex in a transverse hemisection of the caudal forebrain illustrating component nuclei, including descending projections to the brainstem (hindbrain); medial is to the right and dorsal is up. (**b**) A schematic hemisection corresponding to **a** and illustrating expression of a number of transcription factors (Isl1, Nkx2.1, Lhx7, Lhx1,Dll4), neurotransmitters or neuromodulators (GABA, TH,SP and ENK) and a calcium-binding protein (CR). Note the widespread distribution of the inhibitory neurotransmitter GABA within the CeA. Expression of these molecular markers corresponds to similar patterns in mammals (not included). (**c**) A fictive *advertisement call* recorded from the VN after microstimulation in the EA. (**d**) Effective (red, asterisks) and ineffective (black) stimulation sites for evoking *fictive advertisement calls*. (**e**) Comparison of temporal features (fast and slow trill) recorded from an *advertisement calling* male (*in vivo*), a *fictively advertisement calling* male brain (*ex vivo*), and following EA microstimulation (*ex vivo* EA stim) indicates that EA activity can initiate a specific *fictive call* pattern

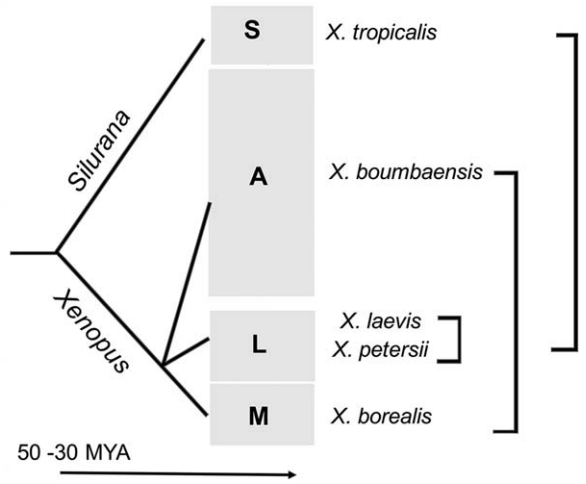

FIGURE 5 Examples of vocal circuit comparisons across and within *Xenopus* clades. The S, L, and M clades use different genetic mechanisms for primary sex determination. Comparison of neural circuit elements—using a fictively calling preparation—across species from these clades should reveal common and diverged neural circuit mechanisms. Divergence time estimates of *Silurana* and *Xenopus* based on Canatella (2015). Phylogeny simplified from Evans et al. (2015)

responsible for behavioral differences. This approach—comparative neuromics—is analogous to the comparative genomics approach used to distinguish functional and non-functional DNA within evolutionarily conserved sequences and to identify some important functional elements. Comparative neuromics has been used at the single neuron and circuit levels level to compare locomotory circuits in gastropod species (Katz, 2016). Here we propose extending this approach to analyze neural circuits that generate vocal patterns (Figure 5). The fictively calling preparation in recently diverged species (10 to 500 thousand generations) should reveal mechanisms for retuning a specific neural circuit component. Over longer time scales (a few million generations), we can determine which elements of neuronal circuitry diverged. Comparisons over time scales of 10's of millions of generations can identify invariant and critical neural circuit components for a specific function (but see Gjorgjieva et al., 2016).

The evolutionary history of the genus *Xenopus* has been estimated using molecular phylogenetics and other evidence. An *advertisement* call is present in males of all extant species and inter-species variation in temporal and spectral features of these advertisement calls have been evaluated in a phylogenetic context (Tobias, Evans, & Kelley, 2011). Comparison of species closely related to *X. laevis* (L clade: Figure 5) reveal that intrinsic differences in the period and duration of activity in one circuit element—DTAM—can account for species differences in call temporal patterns (Barkan & Kelley, 2012). *Advertisement call* patterns within different *Xenopus* clades (A, M, and L) diverge dramatically. Across these clades, the very distantly related species—*X. borealis* and *X. boumbaensis* (Figure 5)—share a temporally simplified *advertisement call* pattern (Tobias et al., 2011). In *X. boumbaensis*, the hindbrain neural circuit has been re-tuned to reduce the duration of fast trill (Leininger et al., 2015). In *X. borealis*, however, the hindbrain neural circuit of males produces a temporally simplified pattern (Leininger & Kelley, 2013), suggesting a global alteration in active neural circuit elements, such as inactivation of DTAM contributions identified in *X. laevis*. Within the S clade (Figure 5), one species (*X. tropicalis*) is in wide use as a biological model for genetics and development (Blum et al., 2009;

Koide, Hayata, & Cho, 2005). Comparison of neural circuit elements—using a fictively calling preparation—across species from the S, A,M and L clades should reveal common and diverged neural circuit mechanisms. Recent evidence from *X. tropicalis*, *X. laevis* and *X. borealis* suggests the operation of at least three genomic mechanisms for primary sex determination, one in the S, one in the L and one in the M clade (Furman & Evans, 2016; Mawaribuchi et al., 2016; Roco et al., 2015; see Figure 5). Retuning in response to hormones can thus also be followed across species with divergent modes of primary sex determination.

7 | *XENOPUS* AND THE BRAIN INITIATIVE

In 2013, the United States launched an effort known as the BRAIN (*B*rain *R*esearch through *A*dvancing *I*nnovative *N*eurotechnologies) Initiative to "accelerate the development and application of innovative technologies . . . to produce a revolutionary new dynamic picture of the brain that, for the first time, shows how individual cells and complex neural circuits interact in both time and space." The potential payoff for this initiative is extraordinary but the challenges are steep. The major hurdles for the BRAIN Initiative are: imaging activity at scale, extracting functional circuit activity and determining the molecular ID of individual neurons in those circuits.

The smaller the brain, the easier it is to visualize all of the neurons responsible for producing a specific circuit function. Neural activity produces transient changes in calcium levels that can be tracked as fluorescent signals using genetically encoded calcium sensors (Nakai, Ohkura, & Imoto, 2001). Imaging an entire brain requires that all neurons express these sensors and this can be achieved via inserting the sensor molecule (e.g., GCaMP) into the genome and restricting expression to nerve cells (Ahrens, Orger, Robson, Li, & Keller, 2013). While some nervous systems such as flies (*Drosophila*) and worms (*Caenorhabditis*) are small enough for whole nervous system imaging (Prevedel et al., 2014), fundamental brain functions in these non-vertebrate models—such as the control of breathing and vocalizations—differ markedly from vertebrates as might be expected from estimates of vertebrate/invertebrate divergence (~600 million years). Within vertebrates, zebrafish (*Danio rerio*) larval escape and locomotion-related neural activity patterns can be imaged *in vivo* with calcium sensors and light-sheet microscropy (e.g., Dunn, Gebhardt, et al., 2016a; Dunn, Mu, et al., 2016b). Imaging in zebrafish is, to date, however confined to larval stages and individual behaviors, rather than social interactions; imaging usually requires experimental immobilization or natural immobility (Muto & Kawakami, 2016). Ventilation via the gills, and the absence of vocalization (unlike some other fish, e.g., Feng & Bass, 2016), preclude zebrafish as a model for understanding linkages between respiration and vocalization.

Xenopus, like other anurans (Gerhardt & Huber, 2002), are vocal. Their small brains generate fictive respiratory and distinct, sex-specific vocal patterns *ex vivo* obviating the need for immobility. The *ex vivo* preparation also offers the rare ability to study forebrain influences on motor patterns. Transgenic lines of *X. laevis* that express a calcium sensor (GCaMp6s) in all neurons (a collaboration with the National Xenopus Resource at the Marine Biological Laboratory) are being generated for evaluation in the fictively calling brain. Recent advances in light sheet microscropy and the post-imaging identification of individual neurons (Tomer, Ye, Hsueh, & Deisseroth, 2014; Tomer et al., 2015) will provide new tools for understanding functional circuits in *Xenopus* not only for motor patterns but also for sensory processing. Whole brain activity mapping in the *fictively calling Xenopus* brain can substantially advance the objectives of the BRAIN Initiative.

8 | COULD A NEUROSCIENTIST UNDERSTAND A MICROPROCESSOR?

In an influential recent post (http://biorxiv.org/content/early/2016/05/26/055624), Jonas and Kording argue, using a microprocessor as a model organism, that currently available algorithms fail to reveal the hierarchy of information processing actually built into the circuit. Our ability to analyze the large and complex data sets supplied by whole brain imaging is limited not only by technological limitations, but also by gaps in our knowledge of the evolutionary breadth of nervous systems. In living organisms, essential components of information processing can be obscured by shared phylogenies. For example, some neurons in a nucleus of the ventral forebrain (the hippocampus) encode a specific spatial location ("place" or "grid" cells). In rodents, the hippocampus exhibits strong neuronal oscillations at 6–10Hz, the theta wave, which has been linked in both rats and mice to memory and navigation (Buzsáki & Moser, 2011). Bats also have place and grid cells but no theta waves (Yartsev, Witter, & Ulanovsky, 2011), suggesting that theta is not essential and that its prominence in mice and rats is due to shared phylogeny rather than conserved function. Thus the comparative neuromics approach described above also has the potential to contribute to understanding which conserved neural circuit elements might be essential for a neural function and which reflect a shared phylogenetic history.

In summary, the fictively behaving *ex vivo* preparation in *Xenopus* provides a rare opportunity to combine recent advances in the analysis of neural circuits—brain-wide imaging, connectomics, molecular identification of single neurons—with comparative neuromics to determine specific functional components. Systematic analyses of the neural circuits underlying vocalizations can identify CNS mechanisms that underlie changes in behavioral phenotypes across evolution.

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