



Translational implications of the anatomical nonequivalence of functionally equivalent cholinergic circuit motifs

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Biomedical research is at a critical juncture, with an aging population increasingly beset by chronic illness and prominent failures to translate research from “bench to bedside.” These challenges emerge on a background of increasing “silo-ing” of experiments (and experimenters)—many investigators produce and consume research conducted in 1, perhaps 2, species—and increasing pressure to reduce or eliminate research on so-called “higher” mammals. Such decisions to restrict species diversity in biomedical research have not been data-driven and increase the risk of translational failure. To illustrate this problem, we present a case study from neuroscience: cholinergic suppression in the cortex. In all mammals studied so far, acetylcholine reduces activity in some cortical neurons. Comparative anatomical studies have shown that the mechanism behind this suppression differs between species in a manner that would render drug treatments developed in nonprimate species entirely ineffective if applied to primates (including humans). Developing clinical interventions from basic research will always require translation, either between species (e.g., using a mouse model of a human disease) or within a species (using a subset of humans as a representative sample for all humans). We argue that successful translation will require that we 1) be data-driven in our selection of species for study; 2) use (with careful attention to welfare) animals that minimize the translation gap to humans; and 3) become agile at translation, by resisting the pressures to narrow our focus to a small number of organisms, instead using species diversity as an opportunity to practice translation.

bioethics | animal research | neuroscience | systems | comparative

At the International Physiology Congress in 1929, noted physiologist and 1920 Nobel Laureate for Physiology or Medicine August Krogh observed that understanding “the essential characteristics of matter in the living state” requires “the study of the vital functions in all their aspects throughout the myriads of organisms” (1). This perspective is essentially a zoological one: scientists must attend to a wide swathe of organisms across the zoological landscape (and seascape) in order to grasp the dynamics and mechanics of living matter. Indeed, later in his address, Krogh notes that in order to identify which organisms to study to address particular physiological, biological, or medical problems of interest, “we must apply to the zoologists to find them and lay our hands on them” (1).

Rehabilitating the Krogh Principle

Yet, too often when scientists remember August Krogh, they remember—or rather misremember—instead a different fragment of his address that day: “For such a large number of problems there will be some animal of choice or a few such animals on which it can be most conveniently studied” (1). This passage was highlighted in a 1975 paper by Hans Krebs (2), the 1953 Nobel Laureate in Physiology or Medicine, and has been misinterpreted ever since. To wit, Randall, Burggren, and French (3) misrepresent Krogh by paraphrasing this passage as “for every defined physiological problem, there was an optimally suited animal that would most efficiently yield an answer,” while Feder and Watt (4) claim that Krogh held that “for every biological question is an

organism best suited to its solution.” These latter interpretations elide the zoological context that is critical to the correct interpretation of the Krogh principle (5).

One effect of this elision is the canalization of research trajectories in the biological and biomedical sciences (6). For rather than collaborating with zoologists to ascertain the right animal(s) for the job at hand, and then justifying that choice of experimental organism, it has instead become commonplace for scientists to study the animal(s) everyone else is studying. Institutional animal research practices are conservative; establishing research programs with unfamiliar animals is organizationally and ethically challenging. Along with funding trends at the National Institutes of Health (in the United States), at any rate, this helps to explain the distillation of research energies into a relatively tiny number of animal species (7–9) which are nowhere near representative of the “endless forms most beautiful and most wonderful” (10) that comprise the natural biodiversity of the zooscape—and the potential experimental matériel of the practicing biologist.

Moreover, one effect of this canalization of research trajectories is the relative inability of biologists to make serious progress—that is, progress corresponding to the massive investments in basic and clinical-translational research by the National Institutes of Health and private funders—toward resolving significant health issues in the United States and globally (11, 12). The failure to translate from bench to bedside remains the rule and not the exception, attrition in drug studies remains exceptionally high, the US Congress is threatening to dramatically reduce funding for nonhuman primate research, and federal budgets for science funding are under siege. The status quo is no longer working, if it ever did. In response, one place to start is to rehabilitate the Krogh principle by resituating it in its zoological context (cf. ref. 13): the research question, and not the conventionally available research matériel, must drive animal experimentation.

The Choice of Experimental Organism

The most straightforward case of experimental organism choice is where the organism itself is the target of interest. A biologist wanting to understand some aspect of mating calls in a songbird

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will select that particular species of songbird for study and then design experiments to elicit some hitherto unknown or unappreciated or underappreciated feature of its mating call. Given within-species variability, the need for extrapolation to conspecifics will remain, but intraspecies extrapolation is typically the easy case. Every other instance of experimental organism choice will be less straightforward, for the organism will serve either as an exemplar of a group or a surrogate for a target (ref. 14, cf. ref. 15). We will consider these instances in turn.

An experimental organism is an exemplar of a group—an exemplary model, to use Bolker's (14) terminology—where it is meant to represent some feature found both in the organism and more broadly in the group. Bolker discusses zebrafish as an exemplary model in developmental biology for vertebrates. There are lots of reasons to work on zebrafish (their relative fecundity, ease of breeding and rearing in laboratories, short lifespan, and transparent embryos, for instance), and multiple laboratories' significant investment in zebrafish genetics has made them easy to manipulate developmentally; for these reasons, they were established, beginning around 1970, as a standard animal in (molecular) developmental biology (16). Structures, features, and mechanisms—genetic, developmental, anatomical, or physiological, inter alia—may be more readily elucidated in zebrafish than in (some) other experimental vertebrates, although they may be replicated or validated in other vertebrates, and biologists can demonstrate the phylogenetic conservation of these structures, features, or mechanisms throughout the vertebrates. Zebrafish are thus exemplary models.

By contrast, an experimental organism is a proxy for another organism—a surrogate model, in Bolker's (14) terminology—where it is meant to stand in for some other organism that is decidedly more challenging to study directly, whether for reasons of convenience, economics, or ethics. Bolker discusses mice and rats as surrogate models in the biomedical sciences, where they overwhelmingly serve as stand-ins for humans. Some of the same pragmatic reasons apply for choosing mice and rats as surrogate models as for choosing zebrafish as exemplary models: their fecundity, relative ease of breeding and rearing in the laboratory, short lifespan, and genetic manipulability. With surrogate models, though, the primary aim is not for them to represent a group as with exemplary models, although they may do so on occasion (14). That said, mice and rats are members of the order *Rodentia*, a diverse order comprising 2,277 species; Krubitzer, Campi, and Cooke (17) maintain that, at least in terms of cortical organization, there could be no such thing as an exemplary rodent. However, a mouse or a rat could serve as a surrogate model for another animal, whether a rodent; another mammal; or, indeed, a human.

An issue that has arisen time and again in the history and philosophy of science literature, as well as in the scientific literature, on the choice of experimental organism is the strength of the justification of the choice (6). As noted above, pragmatic considerations play a key role in experimental organism choice, as they must. Organisms that are unwieldy in the laboratory, that are too long-lived, that produce very few offspring, or that are difficult to manipulate genetically—or all of the above—will generally be poor choices for the experimentalist looking to produce publishable results on a manageable timescale. So too will organisms that are very expensive to breed and maintain, whether economically or ethically.

None of this is to say that in designing particular individual experiments are biologists engaging in local cost-benefit calculations about which animal species to use in order to gain quick, publishable results. Scientists give, as they must, scientific justifications for individual experiments and entire experimental programs. However, scientific choices are nonetheless constrained, including by history (for instance, the choices of previous scientists); economics (for instance, funding agencies' priorities); and

ethics, law, and politics (for instance, the permissibility of research with certain species of animals). No individual scientist proposes an experiment, as it were, out of thin air: she brings her own skills and interests to the bench, within a research trajectory that begins in graduate school. Decisions about which species she studies—or knows how to study—have often already been made long ago. What we are concerned about is the collective impact of all of these decisions, now and in the past, on the current and future shape and scope of scientific knowledge in neuroscience.

In the biomedical sciences, where the ambition is to gain purchase on some phenomenon of direct relevance to human health, often humans will be, epistemically, the ideal experimental organism but will be problematic choices for a number of more pragmatic reasons. Studies over multiple generations take too long, genetic manipulation is generally forbidden, and so on. Tradeoffs are therefore the norm in the pursuit of surrogate models that can satisfy pragmatic demands. However, can they also meet epistemic demands? Can surrogate (nonhuman) models adequately represent humans? A rehabilitated version of Krogh's principle suggests an affirmative response. The vulgar version presently in circulation may suggest otherwise.

Given failures of translation from surrogate models to humans, we cannot take for granted that our current models are the right ones. It may be the case that pragmatic considerations have overwhelmed epistemic ones, such that getting experimental results has, however unintentionally, prevailed over getting results that actually matter. It may further be the case that we have gotten stuck in our models and we cannot escape them and that the models, rather than the questions, have begun to drive the research (6, 11). These hypotheses could help to explain the overabundance of research with mice and rats and the relative dearth of research with other potentially relevant animals, including nonhuman primates, in clinical-translational biology. However, sometimes experiments with nonrodent species are critically important.

Neuromodulatory Control of Cortical Circuits

Taking neuroscience as an example endeavor within the biomedical sciences, we will present a case study on the use of rodents and nonhuman primates as surrogate models for understanding the human brain. Arguably, the purpose of brain cells (neurons and glia) and the interactions between them is to produce behavior that is well adapted to current internal (e.g., hunger and exhaustion) and external (availability of food and shelter) conditions. Like the conditions that drive them, the behaviors produced by the brain must be dynamic, often on a timescale that precludes adding or removing cells and/or their interconnections. One mechanism by which the required dynamic flexibility in the output of brain circuits is achieved on fast timescales is through the release of neuromodulatory molecules such as acetylcholine (ACh), noradrenaline, serotonin, dopamine, and histamine. These molecules take the potential connectivity embodied by the wiring diagram of the brain and specify the moment-to-moment responsiveness of, and functional connection strengths between, network elements.

Neuromodulation is a critically important brain function; 17 of the 20 most frequently prescribed psychiatric medications target one or more of the neuromodulatory systems (18, 19). A challenge that arises in connection to altering neuromodulatory function in a clinical setting is that in many cases we do not know whether these interventions are effective (to the extent that they are effective) because the targeted neuromodulator is itself the site of pathology, or whether their effectiveness arises because seizing control of neuromodulation is simply a very powerful means by which one can modify brain and behavior. To untangle these possibilities and guide the development of more sophisticated and targeted intervention strategies, a deeper understanding of neuromodulatory function is needed.

A Case of Functional, but Not Structural, Equivalence between Species: ACh in Sensory Processing

ACh's role as a signaling molecule in the nervous systems is ubiquitous. First described by Otto Loewi in 1921 (20), ACh is the molecule that carries signals from the vagus nerve to the heart muscle. Loewi called the molecule he discovered *vagusstoff*; it was later determined that this molecule was in fact ACh, identified by Sir Henry Dale in 1914 (21). ACh carries chemical signals between neurons and motor effectors in species across the evolutionary tree, from annelids and nematodes (22, 23) to vertebrates (20). In many (but not all) species, ACh also acts as an interneuronal signaling molecule, and it is in this capacity that it serves as a neuromodulator in vertebrates. While ACh is released across the entire neuraxis, its most intensively studied modulatory functions in vertebrates arise out of its release into cortex.

The vast majority of the ACh released into cortex in rodents, and all of it in primates, comes from neurons whose cell bodies lie in subcortical structures of the basal forebrain (NB/SI in Fig. 1). These forebrain nuclei are often referred to as the cholinergic basal forebrain, but in rodents, fewer than 20% of the neurons that project from the basal forebrain to cortex actually synthesize and release ACh; most of the other 80% make the classical neurotransmitters GABA and glutamate (24, 25). So, in order to understand how the basal forebrain system supports cognition and behavior in the healthy brain, considerable effort has been deployed toward determining markers that allow one to distinguish cell types in the basal forebrain (cholinergic, GABAergic, and glutamatergic), toward understanding the effect(s) these signaling molecules have once released into cortex, and toward determining how those effects might combine to contribute to behavior [see, for example, the review by Jones (26)].

From the perspective of using biological research to improve human health, a problem with these efforts is that we have known since the late 1980s that in the basal forebrain of both human and nonhuman primates, that large population of non-cholinergic projection neurons likely does not exist (27–30; reviewed

by ref. 31). This is a fascinating species difference because, in general, subcortical nuclei, including the basal forebrain, have not scaled along with the expansion of cortex; they have hypo-scaled. That is, over the course of evolution, the cortex has gained neurons to a greater extent than have the subcortical structures that support its function. For the basal forebrain, however, there has been a profound functional hyperscaling of the cholinergic innervation of cortex in primates, compared to other species.

This of course means that rats and mice are unlikely to be suitable surrogate models for primates (human and nonhuman) in studies of the cholinergic system. This does not mean that rodents are not interesting, or useful, models—we will return to this later—but they are not suited to studies in which a key goal is to support translation to clinical interventions directed at the cholinergic system and improving human health. Furthermore, this difference in the chemical anatomy of the basal forebrain is by no means the only documented species difference in cholinergic system anatomy. Intriguingly, structural differences have been identified both within and between species in circuits whose function appears to be conserved.

In 1992, Hasselmo and Bower (32) showed that the effect of ACh release differs between pathways into (afferent) and within (intrinsic) cortical circuits. Recording in the piriform (olfactory) cortex of rats, they found that ACh suppresses activity in intrinsic pathways, resulting in a relative enhancement of the afferent pathway into the circuit. Using pharmacological dissection, they showed that this effect was mediated by the m2 subtype of muscarinic ACh receptor acting to suppress release of the excitatory neurotransmitter glutamate. A few years later, Gil et al. (33) showed that, again acting through m2 receptors, ACh similarly suppresses intrinsic pathways in the somatosensory cortex of both rats and mice. However, their circuit turns out to be a little different from that found in piriform cortex of the same species; in somatosensory cortex, concurrent with the suppression of intrinsic pathways, there is an enhancement of activity in the afferent pathway into cortex, this time mediated by nicotinic ACh receptors acting to increase glutamate release. This of course only serves to strengthen the relative enhancement of that afferent pathway and as such is a functionally (but not structurally) equivalent circuit to that observed in the olfactory cortex. Interestingly, Gil et al. also report that the specific subtype of nicotinic receptor that mediates the afferent pathway enhancement may differ between rats and mice (i.e., that the receptor subtypes are pharmacologically and functionally distinct).

Within 5 years of the Gil et al. study, this circuit motif, in which ACh acts through nicotinic receptors to enhance afferent drive and through m2-type muscarinic receptors to suppress intrinsic activity, had been confirmed for the auditory and visual cortices of the rat (34, 35). Hasselmo and McGaughy (36) went on to propose that the role of cholinergic modulation in sensory cortex is to privilege the processing of data from the sensory world over processing of internal states and that the concentration of ACh in the tissue thus controls the balance between attentive states and memory retrieval.

When groups studying nonhuman primates (specifically macaques and marmosets and macaques) conducted experiments inspired by these data and theories, it was found that in these species, too, ACh enhances afferent drive to sensory cortex (37) and is released into sensory cortex during attentive states (38). The afferent enhancement was confirmed to be mediated by the nicotinic receptor subtype observed in rats (37). Furthermore, although differences in method preclude a direct comparison with the rodent data, and the primate studies differ in their conclusions regarding how widespread the effect might be, it was also found that ACh suppresses intrinsic activity in cortex, via muscarinic receptors (37–41).

Things were thus starting to look very good for a panspecies unifying theory of cholinergic function in sensory processing.

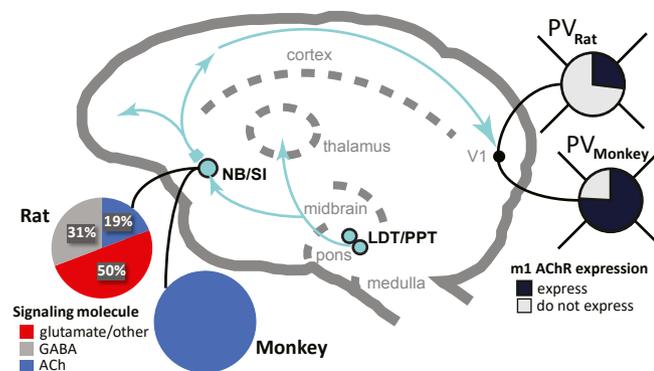


Fig. 1. Key known differences in the structure of the cholinergic system in macaques versus rats. Subcortically, cholinergic neurons are found in nuclei (light blue circles and arrows) of the brainstem (laterodorsal tegmental nucleus [LDT] and pedunculopontine tegmental nucleus [PPT]) and basal forebrain (nucleus basalis [NB] and substantia innominata [SI]). (Left) Cortical cholinergic innervation (blue portion of pie chart) arises from the basal forebrain, which comprises ~100% cholinergic projection neurons in macaques and other primates and only ~20% cholinergic projection neurons in rodents. In rodents the vast majority of the remaining 80% of projection neurons release GABA (gray) or glutamate (red). (Right) In the primary visual cortex of both rats and monkeys, the largest population of inhibitory (GABAergic) interneuron expresses parvalbumin. In macaques (and humans), 75 to 80% of these neurons express the m1 type muscarinic ACh receptor (dark blue in pie chart). In rodents, only ~25% express the same receptor. Data are adapted from refs. 24, 25, 27–30, 44.

There was one problem, however; an anatomical study showed that the m2 muscarinic receptors known to mediate cholinergic suppression in rodent cortex could not be found in the correct circuit position to serve this function in a primate (ref. 42; Fig. 2). In rats and mice, the suppression arises from reduced release of the excitatory neurotransmitter glutamate in the intrinsic connections of cortex. This requires that the m2 receptor be located at or near the site of neurotransmitter release (axon terminals), a localization that is observed for only 2% of release site structures in nonhuman primates (42). Even more interestingly, an entirely different receptor subtype—the m1 muscarinic receptor—is expressed by neurons in macaque cortex that release the inhibitory neurotransmitter GABA (refs. 42 and 43; Fig. 1). In a follow-up study, it was shown that when a cholinergic suppression is observed in macaque, it is mediated by GABA release (41).

These data suggest a profound species difference, in which suppression by ACh is observed in both rodents and primates, but comes about via very different mechanisms. In rodents, ACh suppresses activity by reducing excitation through m2 receptor activation, while in primates, ACh suppresses activity by strengthening inhibition through m1 receptor activation. In a final confirmation of the structural nonequivalence of these circuits, it was shown that while in macaques and humans the vast majority (~80%) of the cells comprising the principal subclass of inhibitory neuron in the primary visual cortex (i.e., inhibitory neurons immunoreactive for the calcium-binding protein parvalbumin) express the m1 muscarinic ACh receptor, only 27% of cells in this cell class express the same receptor in rats (44) and probably in mice also (45). Interestingly, expression of m1 receptors by parvalbumin neurons in guinea pigs is nearly as strong as in (human and non-human) primates, and in ferrets, there is expression at an intermediate level (44). Cholinergic suppression mediated by GABA release has also been observed functionally in cats (46). So, not only does m1 ACh receptor expression by inhibitory neurons differ substantially between species, it does so in a manner that is not simple to predict from consideration of the evolutionary history of mammals.

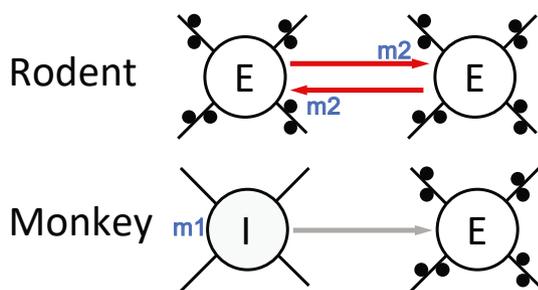


Fig. 2. Cholinergic circuits with analogous function and divergent structure. (*Top*) In the primary sensory cortices of rats, ACh suppresses activity by reducing neurotransmitter (glutamate) release from excitatory neurons (E). This action arises from the activation of Gi-coupled m2 muscarinic ACh receptors expressed by the glutamatergic axons (red arrows), near the site of glutamate release (blue m2). In order to alter the suppressive effects of ACh in these circuits, the m2 receptor or its downstream (Gi-mediated) pathway would be an excellent druggable target. However, m2 receptors are rarely expressed by glutamatergic axons in the primary visual cortex (V1) of the macaque monkey, so a drug so-developed would be ineffective in achieving this goal. ACh can nonetheless have a suppressive effect on neural activity in macaque V1. (*Bottom*) In macaque V1, when suppression by ACh is observed, it is mediated by increased release of neurotransmitter (GABA) by inhibitory neurons (I), the majority of which express the Gq-coupled m1 muscarinic ACh receptor (blue m1) on their soma and proximal dendrites. Thus, the druggable target for cortical suppression by ACh in the cortex of a primate would be the m1 receptor or its downstream (Gq-mediated) pathway.

This is a nontrivial difference; the m1 and m2 subtypes of muscarinic ACh receptor are pharmacologically distinct, activate different intracellular signaling cascades, and generally have opposing effects on neuronal activity. This has profound implications for clinical translation. If it were determined that in order to treat a particular disorder, one would need to alter the cholinergic suppression of cortex, and a novel drug were developed that targets the circuit motif for cholinergic suppression in the rodent cortex (Fig. 2, *Top*), it would be ineffective in targeting the perhaps functionally equivalent, but certainly structurally nonequivalent, suppressive motif in a primate (Fig. 2, *Bottom*).

It is certain that there will be many as-yet undocumented instances of functional equivalence with structural nonequivalence between circuit motifs; organisms that have to solve similar problems are constrained by functional output, not by conservation or replication of extant structure (47). Furthermore, the existence of mechanistic/structural nonequivalences between species likely underlies some percentage of the failure to translate pharmaceutical interventions from rodents to humans. The likely prevalence of this phenomenon should not be underestimated; it has been noted that modulatory receptors have been modified over the course of evolution far more slowly than have the myriad behaviors they support (48). One means by which parallel and/or convergent evolutionary processes can yield similar functional outcomes from distinct mechanistic implementations is through modified receptor expression patterns (48) of precisely the kind we have described here. One does not even have to look to species that differ as much as do humans and rodents to find examples of evolutionary tinkering (47) with receptors; differences in expression of a single serotonin receptor are the likely mechanism underlying differences in swimming behavior between 3 species of marine mollusk (49).

Currently unknown species differences aside, the differences in neuromodulatory anatomy documented so far are profound. Considering just those related to cholinergic modulation of early vision (the research area of one of the authors [A.D.D.]), there are differences in basal forebrain composition (discussed above and reviewed by ref. 31), in the pattern of cholinergic innervation of the primary visual cortex (50), and in receptor expression in the visual thalamus (51, 52), the afferent pathway from the thalamus to the primary cortex (33, 37), and within the intrinsic circuitry of V1 (44).

Yet, despite these profound and widespread differences, the rule of thumb in the field continues to be that one should assume similarity between rodents and humans until proven otherwise, and sometimes well afterward (53). This is not a field-specific bias; a similar assumption, implicit or explicit, applies across much, if not all, of neuroscience today.

The Need for an Agile and Comparative Neuroscience

Researchers who conduct studies on nonhuman primates are held to very high standards for demonstrating the relevance and necessity of their surrogate model, and so they should be. Selection of a rodent as a model in the neurosciences, on the other hand, is often deemed adequately justified by virtue of the fact that rodents are mammals, as are humans. This justification is not good enough; selection of model species for biomedical research should be data driven and either based on known mechanistic equivalence between the surrogate and the human or accompanied (at a minimum at a programmatic level at the NIH, ideally in individual laboratories) by a plan for how the gap between the model and the human is to be translated across.

Jumping across gaps between surrogate models and the human will require translational agility; this can be thought of as a feature of the field rather than being a requirement for individual researchers. Jumping gaps can be made easier in at least 2 ways: 1) by choosing small gaps (for example, based on the above case study, one might choose to study cholinergic suppression of visual

cortex in a primate or guinea pig) and 2) by practicing jumping (e.g., by studying cholinergic modulation of visual cortex in diverse species in a comparative fashion that, over time and across laboratories, yields a deep understanding of the differences that exist and what difference they make). Both small gaps and plenty of jumping practice are likely to be features of a truly agile neuroscience in service of human health.

It should be noted here that the authors do not endorse the view that all biological research need be in service of human health; knowledge has value in its own right. However, the vast majority of public funding for the neurosciences comes from the NIH whose mission is “to seek fundamental knowledge about the nature and behavior of living systems and the application of that knowledge to enhance health, lengthen life, and reduce illness and disability.” The latter part of this mission, and its implied compact with the public whose tax dollars fund our research, carries with it some responsibility for maximizing the utility of our data for the purposes of clinical translation. This does not mean that all research must be focused on translation or have an immediately obvious application. It does mean that due consideration should be given to factors that could influence the extent to which future scientists will be able to apply knowledge gained. The likelihood will be greater if we give more thought to model selection and invest more effort toward understanding how to move knowledge from one model organism to another. That said, not all applications of knowledge obtained through a more comparative biomedical research will require model system agility and translation; veterinary care has also been informed and advanced as a result of animal research. Furthermore, comparative

studies in the neurosciences should extend beyond mammals; many crucial findings in the study of neuromodulation, for example, have been made through study of invertebrates (see, for example, refs. 48, 54, and 55).

Conclusion

In rehabilitating the Krogh principle and expressing the need for an agile and comparative (even zoological) neuroscience, we are not hereby calling for researchers to pick up, or drop, any particular species. Instead, we call for 1) greatly increased diversity of surrogate models, 2) a data-driven approach to selection of models, and 3) a commitment to comparative approaches at the level of individual laboratories and across the field as a whole. This last point is critically important: it is not enough to simply study different species; we must study the differences between species at the structural and functional level. This is what will give us agility in model translation. Then, when we find that differences exist, and if our goal is to improve human well being, there will be times when we need to commit to use a species that actually resembles the human, thereby minimizing the distance across which we have to make that final agile jump to successful clinical translation.

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