PERSPECTIVE

# Tinbergen's challenge for the neuroscience of behavior

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**Nobel laureate Nikolaas Tinbergen provided clear criteria for declaring a neuroscience problem solved, criteria which despite the passage of more than 50 years and vastly expanded neuroscience tool kits remain applicable today. Tinbergen said for neuroscientists to claim that a behavior is understood, they must correspondingly understand its (*i*) development and its (*ii*) mechanisms and its (*iii*) function and its (*iv*) evolution. Now, all four of these domains represent hotbeds of current experimental work, each using arrays of new techniques which overlap only partly. Thus, as new methodologies come online, from single-nerve-cell RNA sequencing, for example, to smart FISH, large-scale calcium imaging from cortex and deep brain structures, computational ethology, and so on, one person, however smart, cannot master everything. Our response to the likely "fracturing" of neuroscience recognizes the value of ever larger consortia. This response suggests new kinds of problems for (*i*) funding and (*ii*) the fair distribution of credit, especially for younger scientists.**

behavior | amygdala | hypothalamus | sex | parental

A problem: Neuroscience is becoming increasingly "fractured." For example, at one extreme, new high-throughput molecular (and computational) approaches to transcriptomic and neuronal activity analyses require full-time efforts. Yet, the underlying goal of neuroscience is to explain behavior and a wide range of cognitive and emotional faculties. So, at another extreme, the experimental skills for running behavioral assays in a rigorous and replicable manner are demanding. One could say that the "fracturing" is an inevitable consequence of the growth and popularity of neuroscience. One nevertheless hopes for as many comprehensive, unifying frameworks as possible.

One such framework, still applicable and timely after more than 50 years, was provided by Nobel laureate ethologist Nikolaas Tinbergen, who wrote that a problem in the analysis of behavior is not solved until scientists understand its (*i*) development, (*ii*) mechanisms, (*iii*) function, and (*iv*) evolution (1).

By "development" Tinbergen did not mean the mere appearance of a behavior, but rather "the changes of behavior machinery during development" (ref. 1, p. 424). These would include mechanisms for imprinting and for early learning. For "mechanisms," Tinbergen primarily seemed to be thinking about them as we would now, except that he could not be as specific as we are now. About "function," he focused on survival of the individual and species. While discussing "evolution" Tinbergen looked forward to studies "with all the methods available in genetics" (ref. 1, p. 428). Especially intriguing for some behavioral neuroscientists are those cases of comparisons between two species in which one important behavior in one species differs from that type of behavior in a closely related species, while a large number of related behaviors remain constant.

Each of Tinbergen's four problems speaks to aspects of current cutting-edge neuroscience. Mechanisms of development of individual nerve cell types are being analyzed with single-cell RNA sequencing (RNAseq) and, in some cases, with the development of organoids. Mechanisms of behavior are now better understood at the single-cell-type level by the use of optogenetics, designer receptors exclusively activated by designer drugs, and other cell-selective techniques. Current understanding of function is not limited to the survival of the normal, typical individual but also to disorders of cognitive and emotional functions. Of course, current uses of molecular genetic techniques offer new approaches to questions about evolution.

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Behavioral neuroscientists have made such rapid strides recently that these four challenges are just now clearly are being met. At the moment, it looks as though the relatively simple behaviors of clear biological importance are leading the way toward fulfilling Tinbergen's requirements. In this perspective we give the primary example of a sex behavior and also include brief examples of work on parental behaviors and defensive responses to threat.

Tinbergen was hard-headed and stood against untestable assumptions about animal mental states. Quoting him (2), "Hunger, like, anger, fear, and so forth, is a phenomenon that can be known only by introspection. When applied to another species, it is merely a guess about the possible nature of the animal's subjective state." The need for objective terminology is recognized now, for example by using the term "defensive response" instead of the word "fear" (3). (We overrode this problem by solving a relatively simple, biologically crucial, and sex-hormone-dependent social behavior as summarized below.)

In this respect, perhaps some of the most exciting and unexpected recent work has uncovered relations between transposable elements and the DNA response elements for nuclear hormone receptors.

## Sex

Here are Tinbergen's four challenges.

***Development.*** The simplest example of a mammalian social behavior is the mating response of female mammals: It constitutes a standing response (following rapid locomotion during courtship) coupled with vertebral dorsiflexion. Called "lordosis," it depends on circulating estrogenic hormones which must enter the brain and bind to estrogen receptor-α (ER-α) in neurons in the ventromedial (VM) nucleus of the hypothalamus.

Ovaries function to secrete estrogens as early as postnatal day 4 with a significant increase starting on postnatal day 7 (4). It is well established that ER-α begins to be expressed on postnatal day15 (5, 6) and, indeed, lordosis behavior is shown first on postnatal day 15 (7). This neuroendocrine system develops further when estradiol, supplemented by progesterone, stimulates the ovulatory luteinizing hormone (LH) surge. Thus, after puberty (days 30 to 36) the ovulatory surge of LH will occur late afternoon on the day of proestrus, well coordinated with lordosis behavior (just after dark) which permits fertilization by the male.

***Mechanisms.*** Mechanisms for mating behavior, obviously a social behavior, in female laboratory mammals have been worked out (8). Lordosis behavior is triggered by somatosensory stimuli on the flanks and rump, usually caused by the male's mounting. Action potentials travel the well-studied cutaneous nerves to arrive in the dorsal horns of the spinal cord at lumbar levels. These signals ascend in spinoreticular and spinothalamic tracts to form a spinoreticulo-spinal loop whose activity is controlled by estrogen-dependent signals from neurons expressing the ER-α in the VM hypothalamic nucleus. Several genes expressed in those hypothalamic neurons have two properties: (*i*) Their mRNA levels are increased in those neurons by estrogen treatment and (*ii*) their proteins produced from those mRNAs subsequently foster lordosis behavior. The estrogen-dependent signal from the VM hypothalamus activates neurons in the midbrain central gray, whose neurons in turn activate the behaviorally relevant reticulospinal and vestibulospinal neurons. They activate the motor neurons for the axial, deep back muscles whose contraction causes lordosis.

This mating behavior, essential for fertilization and therefore for reproduction, is overdetermined in the sense that at several levels redundant mechanisms operate. For example, all of the brain mechanisms are bilateral, and forward of the spinal cord the operation of the lordosis behavioral will survive unilateral damage. Further, it is not just one but several transcriptional mechanisms which are estrogen-sensitive in the relevant VM hypothalamic neurons and involved in fostering lordosis. Also, several neurotransmitters impact VM hypothalamic to excite their electrical activity, again redundant. Finally, progesterone amplifies the estrogen effect.

Of course, there is a massive sex difference in the performance of this behavior. Male mice or rats—or females treated with testosterone during the perinatal period—rarely exhibit lordosis behavior.

***Function.*** The adaptive function of lordosis behavior is obvious. The standing posture coupled with vertebral dorsiflexion allows the male to mount the female and deliver sperm. The redundancy of the neuronal mechanisms enables the individual to mate and produce offspring even in cases of injury or developmental problems.

***Evolution.*** Female sexual selection is a driving force in evolution and lordosis enables internal fertilization, making the ability to perform this behavior crucial to a female's evolutionary fitness (9, 10). Here, the discussion of sexual behaviors of female laboratory animals includes at least three lines of evolutionary thought. During evolution, lordosis behavior co-opted already-developed postural control pathways: vestibulospinal and reticulospinal (11). Further, the evolution of a female-typical mating behavior is neither limited to lordosis itself nor to the evolution of quadrupeds (in that some female birds have a similar standing posture and vertebral dorsiflexion). Thus, lordosis or similar behaviors likely evolved multiple times, suggesting that it conferred a fitness advantage onto the females that possessed this behavior and the males that were responsive to it. It is likely that female choice allowed for an increase in the fitness of the females and their offspring (12).

Second, there was a clear need for internal fertilization to deal with the transition from living in water to living on land. There evolved corresponding changes in egg proteins (13) and changes in sperm. However, there are complexities; some fish use internal fertilization, suggesting that the optimal mating strategy is dependent on multiple environmental factors. This is supported by evidence that (*i*) internal fertilization has evolved more than once and (*ii*) external fertilization has also possibly evolved from internal fertilization (14). While mating strategies vary greatly among species, overall internal fertilization would be expected to increase the importance of female mate choice as a selective constraint on the reproductive fitness of males, contributing to the evolution of lordosis.

Third, the nuclear receptor, ER-α, expressed in VM hypothalamic neurons in a manner essential for female reproductive behavior, is a member of a large family of nuclear receptors. Current thinking states that the ancestral nuclear receptor had a high degree of similarity to ER-α and that the large family of steroid receptors "evolved according to a principle of minimal specificity: at each point in time, receptors evolved ligand recognition criteria that were just specific enough to parse the set of endogenous substances to which they were exposed" (15). New lines of thinking are also considering relationships between the evolution of steroid hormone receptors and transposable elements (16). For example, Testori et al. (17) searched for transposable elements overlapping ER-α binding peaks in publicly available ChIP

sequencing databases and found that "ERα preferentially targets a well-defined set of Transposable Elements (TEs) and that these TEs host combinations of transcriptional regulators involving several of known co-regulators of ERα." Regarding another nuclear receptor, the vitamin D receptor, PCR-based amplification of the Alu short interspersed nuclear element from human and nonhuman primate genomic DNA and subsequent sequence analysis revealed perfect structural conservation of the vitamin D response element (18).

Ultimately, for the mammalian sexual behavior which is the key to reproductive success, the evolutionary question boils down to the evolution of a gene regulatory network. This question has been approached recently (19), but much work along these lines remains to be done.

## Parental Behaviors

Less is known about parental behaviors, which have more complicated hormonal requirements and more complicated sensorymotor topographies than female reproductive behavior. We focus on them here because they constitute natural consequences of sex behaviors mentioned above and because the complexities of their endocrine determinants provide an interesting contrast to the relative simplicity of estrogen-dependent behaviors. For example, lactation depends not only on estradiol but also on declining concentration of progesterone and on high levels of prolactin, corticosterone, and oxytocin. Nevertheless, parental behaviors serve to illustrate the timely application of Tinbergen's four questions.

***Development.*** Among mammals, maternal behaviors can begin as soon as the female can support a pregnancy and a normal delivery, but "primiparous" mothers (females giving birth for the first time) are more susceptible to the interruption of their parental behaviors than females with previous care-giving experience. During development, maternal behavior must begin with nest-building and with pup retrieval. Then, nursing is the most universally expressed maternal behavior among mammalian females (20).

***Mechanisms.*** Classical work by Numan (21) established preoptic area neurons as required for maternal behavior in female mice and rats. In a step toward unraveling the transcriptional steps required to support normal maternal behavior, we showed that the ligand-activated transcription factor ER-α needed to be expressed in these preoptic neurons (22, 23). The full scope of maternal behavior—building the nest, retrieving pups to the nest, licking and warming the pups, and nursing—is facilitated by the neuropeptide oxytocin and transcription of the estrogen-dependent oxytocin receptor. Some recent molecular evidence has implicated medial preoptic area neurons which express galanin (24). Most interesting are the data showing not only that preoptic neurons expressing ER-α are necessary and sufficient for elements of maternal behavior, but also that they electrically activate during pup retrieval and that they drive the behavior in part by connections to a part of the midbrain important for behavioral reward (25).

***Function.*** Clearly, the function of mammalian parental behavior is to enable the young to survive until the age of reproductive competence, thus prolonging the species.

***Evolution.*** The evolution of maternal behavior is no less complex than the evolution of mammals. Recently it has been considered in the light of oxytocin's powerful influences (26), the onset of maternal behavior after adolescence (27), and human attachment (28).

## Defensive Responses to Threat

***Development.*** Neuronal circuit development begins with patterning and subsequent generation of amygdala (early to mid neurogenesis, E11 to E15 in the mouse) and cortical/hippocampal (early to late neurogenesis, E11 to E18 in the mouse) circuit components (29–33). This process specifies glutamatergic pyramidal neurons in prefrontal cortex (PFC), basolateral amygdala (BLA), and hippocampus originating in dorsomedial and ventral pallium through Nkx2, Lhx2/9/7, Lmo1/3/4, and Dbx 1. PFC, hippocampal interneurons, intercalated cells (ITCs), and central amygdala (CE) GABAergic interneurons emerge from ventrolateral and medial ganglionic eminences, controlled by Lhx6, Islet 1, and Dlx5/6. These factors are embedded in complex combinatorial transcriptional networks and mechanisms for generating and differentiating (34) pyramidal (31) and GABAergic interneurons (32). Within these neurogenetic networks, transcription factors may control well-defined neuronal circuit elements for specific aspects of defensive behaviors [e.g., control of ITC fate, extinction, anxiety, and social interaction by Tshz (35) or Maf/Mafb and Dlx1/5/6 specifying BLA $PV^+$ vs. $SST^+$ interneurons (36), circuit elements promoting or dampening in aversive learning, respectively (37)].

During behavioral development from postnatal age to puberty and adulthood, defensive circuities undergo substantial functional maturation. From midinfancy to weaning, animals switch from the paradoxical attraction to shock-paired cues to normal defensive responding by avoiding them. Behaviorally, this delayed ability for forming and expressing such aversive associations facilitates maternal interactions; with higher mobility in older infants, this gives way to need for aversive reactions to heights and strangers (38). This behavioral shift has been linked to maturation of GABAergic signaling in the amygdala (39). Likewise, from weaning to adult age, hippocampal maturation allows one to additionally integrate contextual information to form more complex aversive memories (40) and increased prefrontal-amygdala interconnectedness enhances their extinction (39).

***Mechanisms.*** Since Tinbergen's original proposal, Pavlovian conditioning emerged as the most straightforward and informative proxy into ethology and neuroscience of defensive behaviors in the laboratory (41). Typically, this has been investigated in auditory or contextually cued foot shock conditioning or active avoidance paradigms in which neuronal pathways and mechanisms integrating conditioned (tone-CS) and unconditioned (footshock-US) in training and recall sessions and to switch the animal between passive (freezing) or active (flight) defensive reactions. Pioneering classical functional neuroanatomical lesion studies (42), complemented by recent circuit level opto/pharmacogenetics and optical or electrical activity recordings (37), delineated a circuit framework for what was called conditioned fear behavior. A network of glutamatergic projection neurons and $PV^+$ and $SST^+$ interneurons in BLA integrates conditioned stimuli (CS; e.g., tone) from auditory cortex and aversive events (unconditioned stimulus, US, e.g., foot shock) from thalamic and sensory cortex inputs to form the appropriate memory. These signals are relayed via CE lateral nucleus (CEl) inhibitory networks (43, 44) [e.g., CEl $SST^+$ (45) and $PKC\delta^+$ (46) neurons and central medial output neurons] to brainstem circuits that control defensive behaviors (e.g., freezing) (47). This canonical information flow is gated in the BLA by ITCs and medial prefrontal inputs (48) and mixed with contextual

information from ventral hippocampus (49). In addition, BLA signals are modulated further in CE, which integrates additional CS and US signals from paraventricular thalamus (PVT) (50) and lateral parabrachial nucleus (51).

At the cellular level, the Pavlovian associations are integrated at synapses to BLA pyramidal cells, controlled by homo- (52) and heterosynaptic long-term potentiation (53) and postsynaptic glutamate receptor trafficking (54). Theses associative synaptic memories in the BLA–CE network are reinforced by dopamine dedicated midbrain systems (55) assembling into discrete network engrams (56) of aversive experiences (57). "Fearful" stimuli and behaviors are represented in distributed neuronal activity of neuronal ensembles (58). This local activity synchronizes between amygdala and hippocampus (59) and PFC and amygdala (60) during induced defensive response states. Such timing may facilitate coupling dynamic states and information flow across the network which regulates defensive responses.

At the molecular level, activity of this network is strongly modulated by cannabinoids which control defensive responses in CE circuitry (61) and extinction in BLA (62) circuits. Moreover, signal processing in BLA, ITCs, and CE is modulated by stress-related neuropeptide S (63), neuropeptide Y (64), cholecystokinin (64), corticotropin-releasing hormone (65), and oxytocin (66) neuropeptide systems.

Perhaps not surprisingly, the systems that mediate these phasic conditioned defensive behaviors toward specific threats are also key elements for controlling nonconditioned tonic defensive behavioral states and anxiety. These states are thought to prepare the animals for potential dangerous encounters in ambiguous settings, in the absence of immediate threats (65). These behaviors are typically measured in arena-based assays not primarily scoring active–passive responding but rather a general behavioral bias of the animal's exploratory drive versus its innate aversiveness toward open, unprotected spaces. Specifically, a network in lateral septum, PVT, nucleus accumbens, hypothalaums, ventral hippocampus, and extended amygdala structures (BLA, CE, and bed nucleus of the stria terminalis) forms a core network receiving prefrontal top-down control and gating defensive behaviors through brainstem interactions (67). An emerging theme is that conditioned and unconditioned defensive behavioral states might be multiplexed in these circuits by facilitating defensive neuronal activity pattern and behavioral responding through tonic neuropeptidergic modulation (68–70).

Taken together, the past decade delineated the basic neuronal circuit architectures, dynamic phenomena, and cellular-level mechanisms underlying aversive memories and defensive behavioral responding.

***Function.*** The fundamental function of defensive response to threat lies in protecting the individual from danger. Therefore, the goal of defensive behavior is to safely cope with a threat, which in its most basic form is to freeze to avoid detection. However, the appropriate response strongly depends on threat proximity and spatial context. Thus, defensive behavior ranges from neutral for very distant threats to freezing, escape, and fighting. We have recently begun to understand how defensive circuitry controls these switches between active and passive responding (71–73) and how such behavioral hierarchies are embedded in network activity (68, 73, 74).

***Evolution.*** Both invertebrate and vertebrate organisms have some form of innate and conditioned aversive behaviors [behavioral survival systems (41)]. While the direct homologies are difficult to draw, invertebrates have neural circuit architectures [mushroom bodies in *Drosophila* (75)], and cellular, synaptic, and molecular substrates for aversive associative memories. Among vertebrates, the phylogenetically older systems hippocampus and amygdala are present in reptiles and birds, and avian pallial structures and mammalian cortex have similar wiring (76, 77). Thus, it is likely that homologous wiring motifs supporting learning of defensive responses are shared in these brains, albeit with different gross anatomical organization (78). Within mammals, amygdala circuitry is the central hub for defensive response regulation from rodents to primates and humans. However, the increasing complexity of PFC evolutionary expansion and specialization might involve more complex embedding of emotional response processing within higher cognitive control, even further expanded by verbally expressed concepts (78).

## Ways Forward

Going forward, two approaches to the scientific scene envisioned above are complementary to each other. First, the universe of behaviors as fully understood as those reviewed above must expand. Relatively simple "instinctive" behaviors, as covered briefly above, are biologically crucial and interesting in their own right but comprise a tiny fraction of the full universe of behaviors, even those of simple laboratory animals, not to speak of humans.

Second, investigations of Tinbergen's four problems, each of them demanding and current, have brought current neuroscientists to a broad range of new techniques, for example retro translating ribosome affinity purification, single-cell RNAseq, in vivo single-cell two-photon and calcium imaging, dynamic circuit modeling of fMRI data, mathematical analyses of electrophysiological data and ethological data, and many others. If these various approaches, combined with more traditional approaches, constitute "levels" of analysis, then bridging levels presents another set of challenges.

Of course, several of these new technologies lead to the generation of large datasets, requiring specialized knowledge not only to design experiments and but also to analyze results. For example, as in vivo real-time whole-brain imaging at cellular resolution begins to come online, handling the big data in behavioral neuroscience and the corresponding necessary human resources are expected to be serious issues, which only can be solved by inter- and multidisciplinary approaches including machine learning. Further, it is well understood that behavioral mechanisms involve not only connections between neurons and their patterns of activation but also patterns gene expression of these neurons and their responses to neuromodulators and neurotransmitters, not to mention the effects of their local blood supply. Likewise, those behaviorally relevant mechanisms involve the states of adjacent glial, immune, and other supporting cells involved in enabling the relevant neurons to function properly. With respect to the first point above—the expanding universe of behaviors under serious study—their mechanisms and their interactions with environment correspondingly become increasingly complex, but for Tinbergen's four questions we nevertheless must be able to analyze gene expression, protein modifications, neuromodulatory states, and neuronal activation to create a comprehensive understanding of the behaviors in question.

***Here Is the Point.*** As the number of data points increases exponentially, the complexities of data interpretation will require an intimate familiarity with the techniques used to generate each

dataset to minimize both type I and type II errors (i.e., to avoid both false positives and false negatives, respectively). For instance, the specialized knowledge needed to interpret single-cell gene expression properly is quite different from analyses required to interpret the outputs of electrophysiological or calcium imaging methodologies, which in turn are different from understanding fMRI data. Thus, to integrate these different methods of understanding behavior, in Tinbergen's sense, into comprehensive, behaviorally relevant models of brain function, the expertise of multiple investigators, working together in large teams with modelers and computational biologists would be an ideal arrangement.

In other words, all this may best be accomplished in formally constructed, large multidisciplinary consortia which are dedicated to sharing data for specific, well-defined purposes. Because of the wide distribution of skills required to meet Tinbergen's challenge, ever larger consortia of scientists will be required to form and work together successfully.

***Funding.*** Funding bodies must present more avenues to the necessary support, avenues which encourage the formation of huge consortia by recognizing the facts and perspectives presented here and making sure that consortium leaders are not burdened with impossibly complex application requirements.

***Credit.*** Likewise, the existence of large consortia will necessarily change how individual scientists are recognized in such situations. To enable successful interdisciplinary cooperation, authorship positions on papers must be deemphasized and recognition must be allocated based on the unique role and experiences of the individual within the consortium. It is particularly important that the roles and contributions of young scientists not be undervalued because those scientists are middle authors.

This is not to ignore the attractiveness of intensive cross-training and subsequent multidisciplinary neuroscience. Yes, insight often comes from individuals who are trained in multiple disciplines and who are able to synthesize multiple levels of analysis and technical approaches. However, to be realistic about the potential limitations of individuals, even the smartest individuals, to absorb a large fraction of the skill set which will be demanded for cutting edge work in this rapidly growing field, the need for efficient consortium funding will become ever more obvious. Also, of course, observations offered here about ways forward for neuroscience apply in various measures to some other sciences as well.

Linking, again, to Tinbergen's four problems, recent progress in genetic neuroscientific technology has begun to piece together the development of wiring and architecture of neuronal circuitry underlying basic behavioral patterns and hierarchies. At the mechanistic level, current progress is fueled by, for example, single-cell profiling and optogenetics, which will lead to deeper understanding of neuronal types and circuit elements and regulation of ever more complex behaviors. At the functional level, current advances in computational ethology will lead to a more fine-grained description of behavioral states (79) and with an ethological point of view inevitably will implicate evolutionary thinking. However, most of past and current research has focused on the organization within each of Tinbergen's levels. We have only begun to tackle future challenges in identifying the functional organization across these levels (80–84). This will require combined experimental and computational strategies for mining such functional patterns across genomic, brain architecture, and behavioral datasets (81, 84). These efforts should identify "functional modules" that link genetic programs to development and function of circuit motifs and, ultimately, behaviors. Where are the constraints and the degrees of freedom for genetic variance shaping the evolution of behavioral traits within and between species? With Tinbergen in mind, we ask how these features allow for evolution of behaviors in ecological systems.

Overall, one can envision that neuroscience will move forward in at least two modes of operation which could facilitate each other. Hypothesis-driven research might well proceed in small laboratories, but "hypothesis-free, discovery driven" research yielding big data, as mentioned above, will need the consortia with accompanying accommodations mentioned above.

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