Commentary

MOLECULAR METABOLISM

New horizons for future research — Critical issues to consider for maximizing research excellence and impact

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1. PROLOGUE

We live in an era in which the pace of research and the obligation to integrate new discoveries into a field's conceptual framework are rapidly increasing. At the same time, uncertainties about resources, funding, positions and promotions, the politics of science, publishing (the drive to publish in so-called high-impact journals) and many other concerns are mounting. To consider many of these phenomena in depth, a meeting was recently convened to discuss issues critical to conducting research with an emphasis on the neurobiology of metabolism and related areas. Attendees included a mix of senior and junior investigators from the United States, Latin America, and Western Europe, representing several relevant disciplines.

Participants were initially assigned to small groups to consider specific questions in depth, and the results of those deliberations were then presented and discussed over several plenary sessions. Although there was spirited discussion with sometimes differing

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opinions on some issues, in general there was good consensus among individuals and the various groups. While the discussions were wide-ranging, we have condensed the topics into three (albeit often overlapping) major areas:

1) General research issues applicable to multiple areas of translational research; for instance, animal models, sex and gender differences, examples of emerging technologies, as well as the issue of data reproducibility and related topics.
2) Funding issues, such as how to secure industry funding without compromising research direction or academic integrity, and the training of students and fellows, with a focus on how to optimally prepare trainees for the diverse potential career paths available.
3) Finally, specific research topics of interest were discussed, including whether peptides or other signaling compounds, or specific brain areas, have "thematic functions" or the challenges associated with investigating the function of G-protein-coupled receptors (GPCR) in the brain.

We consider each in turn.

2. GENERAL RESEARCH ISSUES

2.1. The selection of animal models

One of the first questions considered was how good or bad are our current experimental models? As might be expected, discussion initially focused on rats vs. mice. Mice have many obvious advantages including size, cost per animal, a large genomic database, readily available genetically modified strains, and the ability to use smaller amounts of expensive, hard-to-get experimental compounds. On the other hand, rats perhaps have more translational value because they are often better models for human systems and behavior. For instance, most commonly used laboratory rats (Sprague Dawley, Wistar, Long—Evans) are outbred strains and hence have considerable genetic variation, a feature which for many research questions better represents the genetic heterogeneity and diversity of humans. In addition, in certain situations such as after gastric bypass surgery, rats may better model humans because, similar to humans, the substantial reduction in body weight after gastric bypass surgery is mainly due to a reduction in food intake. In mice, on the other hand, food intake is often scarcely changed after gastric bypass surgery, and the reduction in body weight is largely due to an increase in energy expenditure (for review see [5]). Rats have also contributed to a large and rich experimental database and historic development of scientific theories, especially in behavior, physiology, and brain structure.

Given technological advances in molecular genetics, it may be that the 'genetic manipulation' advantage offered by mice will soon be available - at least to some extent - for rats and other, larger, mammalian species that better model certain features of human physiology and behavior. This is a key factor as many systems remain difficult to assess at the desired level in rodents. Nevertheless, public concerns about the use of invasive experimental methods and, in particular, about performing genetic manipulations in animals larger than laboratory rodents that are phylogenetically closer to humans than mice and rats may hamper the use of such animal models in science. This also relates to the question of whether we should always use the best animal model for a given pathology or whether we should compromise with a species that is more accepted for ethical reasons and perhaps even less expensive?

An important concern for much current research is "translationability" i.e., whether what is found in one species (e.g., rat) is also true of another (e.g., mouse, human). How does this impact or create unnecessary redundancy on the one hand and reduce the likelihood of obtaining funding on the other? For example, if one group reports a phenotype in the mouse, and a researcher using a rat model has the means to extend the findings in a novel way, must s/he first demonstrate the basic phenotype in the rat? Many felt that reviewers demand this intermediate step; i.e., it is widely recognized that there is a concern for cross-species validation that must be considered. And while the goal of such research could be justified as comparative physiology, the actual goal is often more closely aligned with issues of modeling and which species more closely resembles human physiology.

In any case, interfacing well with reviewers (of grant proposals or manuscripts) requires strong justification for any model system. It was the group's consensus that the primary scientific concern should be the significance of the research question being asked. There are no good or bad models per se, but there are better or worse models for a particular question, meaning that the value of the model depends on the nature of the question. There should be well-defined criteria to justify the choice of any model. In this climate of shrinking extramural funding, the choice of one model or another must be clearly laid out for reviewers of research proposals as well as for manuscripts, and journal editors should pay particular attention to these issues.

For translational research, a possible strategy would be that journals and funding agencies could include a section detailing the use and choice of the model and how it relates to human physiology if appropriate. Due to space constraints, such sections could be included in the online supplementary material to allow the authors to offer a detailed explanation of the proposed or used model system, including its strengths and weaknesses. Such an approach would, over time, hopefully generate a consensus or at least partial agreement on the applicability of certain model systems to specific research questions.

There was considerable discussion about the utility of other experimental models, including dogs, pigs, non-human primates, non-vertebrates, and computer models. Many of the trade-offs when using these models are obvious. For example, while non-human primates can model humans more closely than rodents, costs, ethical, cultural, and political issues can make such research prohibitive. Differences among rodent strains are just as likely to be as important as those between any species (e.g. [3]). For some less common models that can be justified for particular questions (for example pigs or other large animals), a strong case can be made for collaborating with researchers in animal science, who generally have access to better facilities in which to conduct such research. On the other hand, for more primitive animal models, such as zebrafish, *C. elegans* and other smaller animals, teaming up with specialists in biology may be a viable option. An excellent, recent review summarizes the strengths and weaknesses of currently used animal models [4]. In general, computer models were deemed to still be somewhat limited for addressing research questions in whole-animal physiology and behavior. On the other hand, they may be useful for specific purposes depending upon what is being modeled. Examples include computational modeling of molecular docking and molecular dynamics in drug design to explore the structure and function of diverse therapeutic targets, or, at the other end of the spectrum, simulation models of obesity trends with a focus on the effects of possible policy interventions on public health and economic outcomes.

The point was made that the use of experimentally modified genes in rodent models is now so common that scientific review groups (e.g., at NIH) routinely assign much lower priorities to proposals that simply describe new phenotypes of genetically modified species. Rather,

specific questions regarding gene function need to be addressed which will benefit from the experimental model of genetic modification. It was noted that industry often takes a different approach to animal models, where their goal is not necessarily to understand a system but rather to perform discovery work that leads to marketable drugs or other products. This aspect of the translation issue is often of ultimate importance: How do such data predict human responses?

2.2. Sex and gender differences

The impact of certain research directives mandated by the NIH and other funding agencies, some of which require researchers to design and conduct experiments in a prescribed manner, was another continuing theme in many discussions. For example, NIH's policy requiring justification for using one or both sexes in research raised several concerns. Some felt that this requirement saps limited resources by "forcing" experiments that are not hypothesis-driven, and may not generate important and/or relevant findings.

While investigating sex as a biological variable might be fruitful, it requires careful experimental design to ensure that the studies are adequately powered and data analysis is based on a solid knowledge of genetically- and hormonally-mediated physiological and behavioral differences between the sexes. Studies in females need to take into account the 4 stages of the estrus cycle and, as such, can result in the need for many more animals being studied, including even an ovariectomized group. Many studies are now including both sexes, but the experiments are not always designed to reveal potential sex differences.

Group discussants recognized the value of focused, hypothesis-driven research on sex differences, and suggestions were offered to improve the science being conducted while remaining compliant with the funding mandates. For example, the NIH could provide funding through which graduate students and postdoctoral fellows could be trained in labs that specialize in studying sex differences, and thus, know how such studies should be conducted [e.g., [1,6]]. As sex as a biological variable is a key part of a recent NIH initiative to enhance reproducibility through rigor and transparency [see [2]], perhaps NIH could call for additional proposals that specifically focus on revealing potential sex differences. Other suggestions were 1) to have funding agencies provide supplemental funds for expanding already-funded research to include both sexes, 2) To focus on critical developmental stages that might enhance sexual dimorphisms (e.g., puberty, menopause) when there is likely an important difference, and 3) to fund key exploratory experiments in a "look see" approach to determine the effect of sex in established fields whose findings are largely based on males. The overall point is that many researchers now conduct such experiments in order to be compliant, but they actually have little or no interest in sex differences per se and no pertinent knowledge.

2.3. Examples of emerging technologies

Many topics were considered, although in depth discussions occurred for only a few. The following paragraphs reflect an extended summary of one topic that generated particular interest. There was considerable discussion on the use of designer viruses to define neural networks and investigate their functional architecture. A show of hands revealed that there was widespread use of viruses by the discussants, in part because they are relatively inexpensive to use, are readily available, and provide important anatomical specificity within the nervous system. However, there are often strict biohazard regulatory issues requiring adherence for some viruses.

As with all aspects of research, it is important to know the specific question being asked and whether use of a particular virus is appropriate. In this regard, it was emphasized that viruses can be divided into two general categories — replication-competent strains (such as pseudorabies virus which is used for tracing multisynaptic pathways) and replication-incompetent strains (such as recombinant adeno-associated virus and lentiviruses expressing cDNAs encoding light-sensitive channels, calcium sensitive fluorophores or any other protein or shRNA). Replication-incompetent strains that are broadly used as expression vectors are generally considered harmless. In both of these categories, it is essential to consider the biological properties of the reagent that is to be employed in the experiments and how they will impact upon the interpretation of the data that are produced. For example, the virulence of infecting, replication-competent virus strains has a clear impact upon the specificity of transport through synaptically linked populations of neurons as well as the function of infected neurons within the circuit. The strains of virus most widely used for circuit analysis have been genetically modified to reduce virulence without compromising invasiveness. Nevertheless, these viruses still evoke an immune response in the nervous system that will ultimately compromise the function of infected neurons. Thus, temporal analysis of viral invasiveness of a circuit is an essential component in evaluating both the organization of the circuit and the function of its constituent neurons. There was also concern of toxicity of genes that were cloned into viruses. Fluorescent proteins themselves may generate an immune response and be toxic when overexpressed. Short hairpin RNAs (shRNA), which are used to silence gene expression, may saturate the cellular RNAi machinery such that endogenous miRNAs are not processed properly, necessitating the use of both scrambled compounds and non-injected animals as proper controls to interpret the results in physiological and behavioral experiments, particularly when using adeno-associated and lentiviruses.

The direction of transport of viruses through a neural circuit is also an important consideration in experimental design. Well-characterized strains of viruses have been generated that not only have reduced virulence but also travel selectively either retrogradely or anterogradely through a circuit. Many of these reagents are available from individual investigators as well as through an NIH-funded center headquartered at the University of Pittsburgh (Center for Neuroanatomy with Neurotropic Viruses or CNNV; http://www.cnnv.pitt.edu). The CNNV also provides resources to aid in experimental design as well as access to reviews characterizing the strengths and limitations of the technology. There was a clear consensus among discussants that it is incumbent upon the investigator to become informed on the many issues that impact upon successful application of this demanding technology. Taking advantage of resources available from investigators expert in the technology, as well as those available through the CNNV, can help enormously in achieving that informed perspective.

Increasingly, replication-incompetent viruses and expression vectors are being combined in individual experiments in order to identify the connections of functionally defined populations of neurons. These reagents are mostly employed to highlight connections to a defined population of neurons or to restrict transport of virus through a single synapse. Once again, the biological properties of the viruses used and the ability to alter their genomes are foundational to these powerful approaches. Alpha herpesviruses (DNA viruses) have been most widely used to define the connections of individual populations of neurons within a larger network, whereas rabies viruses (RNA viruses) are employed to define single orders of synaptic input to identified neurons. In both instances, the ability to alter the viral genome to express unique reporters of infection, as well as proteins that influence the invasion and transport of the reporter viruses, have created the foundation for the successful development and application of these

experimental approaches. Discussion of the strengths and limitations of these approaches highlighted the importance of defining the full extent of the neurons whose connections are being investigated. For example, if a neurochemically defined group of neurons is the target of analysis, do all of those neurons become infected with the virus or the expression vector? Also essential is to carefully consider the cytoarchitecture of the injected region and topographical distribution of the targeted neurons within it. Failure to consider these and other important issues can lead to unwarranted conclusions regarding the connectivity of the circuitry under study.

Another approach that is increasingly being applied in the field uses unique fluorescent probes to identify and quantify multiple RNA species (multiplex analysis) in cell cultures or in tissue sections. The capability to assay the simultaneous expression of multiple genes in single cells is very powerful, and several of these methods are currently in use. However, the fact that some are only available commercially can raise problems because reagents are expensive and proprietary, meaning their identity and compositions are not openly available. To offset these issues collaborative networks among labs have formed to help troubleshoot and circulate alternative approaches among those with similar interests. As with all mRNA hybridization methods in intact cells, there are always questions of quantifiability and whether or not one is measuring functional mRNA from which bioactive proteins can be translated. These uncertainties carry the risk of misinterpreting data; for example, the temptation to use changing mRNA levels as proxies of altered protein function.

2.4. The value of replicating published results- the value of failing to replicate published results

The issue of labs failing to replicate what other labs have reported generated lively discussion. Discounting instances of fraud or simply poor training or practice, the discussion settled on 'good' science, why the incidence of failure to replicate is so high (e.g. see [8]), and possible underlying causes. There is, of course, always something to learn from differing results because when both sets of experiments are reliable within one lab or setting, differences between labs likely indicate that a significant, biologically-relevant and as yet unidentified variable (e.g., different strains of subject, different food, different temperature or other lab conditions, etc.) has been overlooked. Importantly, failures to replicate findings are relevant for both *in vivo* and *in vitro* research. Hence, problems to replicate are not a reason to replace *in vivo* with *in vitro* experiments, which is an argument often used by animal protectionists.

At another, perhaps subconscious, level there may be conflicts of interest; i.e., there is often pressure to obtain certain data in order to publish or to secure research funding or a job or promotion, which may prompt a researcher to be less critical than she/he should be or to publish data prematurely, i.e., without sufficient replications. Also, there may be a commercial advantage to promoting one finding over another. In some countries, authors receive monetary bonuses for publishing in high-impact journals (https://www.nature.com/news/don-t-pay-prizes-for-published-science-1.22275), or one's salary may even be directly proportional to one's publishing record (http://www.sciencemag.org/news/2017/08/cash-bonuses-peer-reviewed-papers-go-global). The point is that failure to replicate can be a complex issue, and we often do not invest sufficient resources in determining the underlying cause.

An extension of a lack of replication, especially in some fields, has been the failure of clinical studies to find therapeutic value for drugs that work quite well in animal models. Although the focus of such failure is currently to lay the blame on animal models, it should be noted that clinical studies also suffer many shortcomings in experimental design. More germane to the lack of replication is the large number of fundamental differences that occur in the design and execution of basic versus clinical studies from statistical handling of missing or uncertain data to constraints from ethical guidelines.

In practice, the first published report of a new finding or phenomenon—particularly if it is in a high profile journal— acquires a certain de facto power from its originality or novelty. This sets a standard against which apparently contradictory reports must be judged for publication. While novelty value is obviously important in science, reports of apparent failures to replicate, when these occur, may consequently have to attain a higher bar for publication, even when their methods are appropriate and rigorous.

Novelty and reproducibility can be reconciled more easily by including as much detail about the methods as possible. Over time, when several papers have addressed the same issue, meta-analyses of the published data may be useful, but such results often are not conclusive. Whatever the cause, it is important to include as much methodological detail as possible in original research reports. But even this can be difficult given the way that some journals impose space constraints or relegate methods details to supplementary materials, which are easily overlooked or disregarded. Some journals (e.g., Journals of the American Physiological Society, BioMed Central, the British Pharmacological Society, the Nature Publishing Group, Physiology and Behavior, PLOS, and others) request that all animal experiments should comply with the ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) or the National Institutes of Health Guide for the Care and Use of Laboratory animals (NIH Publications No. 8023, revised 1978). Many journals also endorse the completion of a checklist of critical factors that might affect data validity and robustness (https://www.nature.com/news/surge-in-support-for-animal-research-guidelines-1.19274). However, these endorsements alone apparently do not improve reporting [7], suggesting that the journals should not only support, but more actively enforce adherence to such good practice in publishing. One reason for the lack of adherence may be that complying with these requirements sometimes conflicts with the word or character limit of the manuscript. In any case, adhering to these guidelines might improve researchers' ability to parse out methodological possibilities that underlie differences in results, and it is desirable that publishers, academic societies and funding agencies will soon reach consensus. Nevertheless, and perhaps most importantly, we believe it is our responsibility as scientists to treat each report as a historical record of what took place in a specific set of circumstances. In other words, no single report should be treated as a correct or incorrect finding, but rather as a record of history. The point is that a failure to replicate does not necessarily imply that the initial paper was incorrect. Rather, the implication is that unknown factors are likely at play, and that further attempts at replication from other, independent groups, will be informative.

2.5. Unconscious bias

One perhaps underestimated factor that may contribute to the generation of irreproducible results is unconscious bias. Everything we do is subject to unconscious bias, and it is necessary to be aware of this in order to limit or possibly prevent it. This bias is based on our experiences, culture, prejudices, and many other factors, and it can manifest when designing experiments or interpreting results as well as when reviewing manuscripts or grant proposals. It is occasionally reflected in semantics, when scientists unconsciously state that they perform an experiment to "show something" instead of examining a question or testing a hypothesis. Unconscious bias is difficult to control, but some

guidelines are available (https://royalsociety.org/~/media/policy/Publications/2015/unconscious-bias-briefing-2015.pdf). The Royal Society suggests utilizing some key action points to deal with unconscious bias: 1) when preparing for a committee meeting or interview, try to slow down the speed of your decision making; 2) reconsider the reasons for your decision, recognizing that they may be post-hoc justifications; 3) question cultural stereotypes that seem truthful; 4) remember you are unlikely to be fairer and less prejudiced than the average person; 5) you can detect unconscious bias more easily in others than in yourself, so be prepared to call out bias when you see it.

3. FUNDING AND TRAINING ISSUES

3.1. Funding

An important discussion question concerned ways to secure industry funding without compromising research direction or academic integrity. Several models that are currently working well were discussed. For example, several companies have formed collaborative funding foundations within local communities that include a number of academic research institutions, providing funds to be used for general areas of interest to them and for which faculty from the various institutions can apply. Likewise, similar foundations are funded by groups of philanthropists.

Important issues to consider relate to who owns the data and publishing rights, what are the indirect costs, and whether or not patents might arise. The percent of any profits that accrue to the PI or the PI's lab differ dramatically among institutions, with examples ranging from 10 to 90% being given. Can or should graduate students be recruited to work on industry-funded projects for which proprietary issues may preclude timely publication? It was clear that different institutions and investigators take quite different approaches when addressing these issues.

In addition to contacting a company's research and development department, it was suggested that academic researchers seeking support for early-stage investigations might market their specific abilities, techniques, newly minted molecules, or genetically-modified mice that could be of special value to the company. Further, researchers might propose to study or utilize a product that the company is already developing or marketing, in which case prospective funding may be more forthcoming from the company's marketing division as opposed to its R&D branches.

A quick survey of the meeting's participants indicated that ~75% currently enjoy or have used funds from industry in the past. There was no apparent opposition to the use of such funds, but it is increasingly difficult to obtain funding from industry for basic research without constraints, particularly related to the ability to publish obtained findings.

3.2. Training

Pertinent to interactions with private entities, there was discussion of how doctoral students are being trained. In point of fact, given the current poor prospects for jobs in academia, many of our PhDs will end up in non-academic (or non-research) jobs, and a key question is whether or not we are training them properly for those markets. Examples of non-traditional career paths taken by newly minted PhDs or post-docs include positions in administration, law, business, scientific writing, teaching, the government, non-governmental organizations, and many others. It was mentioned that a recent survey by NSF found that ~70% of the current forty thousand or so PhD students in sciences in the US anticipate doing post-docs when they complete their degree. Students need to be assured, however, that it is acceptable for them to aspire to alternative occupations. It is clear that there are not that many post-doctoral positions available (especially to newly minted PhDs), and that, in many cases, post-doctoral training is unnecessary for the pursuit of alternative non-research-based occupations. As a result, many PhD students will have to, and should, go into these alternate career pathways.

Another and perhaps more problematic bottleneck in academic career paths is finding a position at the assistant professor level. A general consensus was that much of the current graduate training is overly technical and not sufficiently conceptual. So, a key question is, are we training our students appropriately to ensure they are aware of, and competitive for, the wide variety of potential non-academic occupations?

Examples of current strategies and policies that might address these issues include: 1) professional societies or organizations could have more diverse job fairs or clinics, and more informed position listings on their websites; 2) universities could offer specific graduate courses or seminar series that address alternate careers for scientists; 3) graduate programs could include requirements for grant writing and other duties of faculty, put students on department committees, and so on, as these are general skills that are applicable to academic as well as non-academic jobs; 4) industries could establish more apprenticeship programs for PhD students with universities if the funding can be worked out.

One issue that interacts with student training is that, from a mentor's point of view, research has to be completed in order to secure funding, publish papers, advance student careers, and so on. If students are spending large amounts of time on alternative career building activities, it can dilute the mentor's efforts to move projects forward. Because of this, there is considerable variance among mentors and their approach to having students acquire broad skills. In any case, one major goal of a PhD program should be to train the students in "critical thinking" and to emphasize conceptual training (which will be broadly applicable to multiple career paths) in addition to technical training. An interesting possibility is to encourage industrial partners to participate in teaching activities. This could be leveraged (as currently occurs at several institutions) by inviting speakers from industry to PhD program events. Alternatively, it could be beneficial for students to participate in internal training programs (i.e., the Novartis program in drug discovery) which would help educate students about the structures and approaches used in industrial research and development.

4. SPECIFIC RESEARCH TOPICS OF INTEREST

As might be expected, myriad specific topics were suggested for discussion, and we therefore highlight a few areas that were broadly considered.

4.1. "Thematic functions" of peptides, signaling molecules, or specific brain areas

There is a historical notion that one or another peptide (or other compound) which acts at one or more receptors in different systems and tissues can be considered to have an overall "thematic" or interrelated function; i.e., the notion that all of its diverse actions can be related to one over-arching goal (effect) was discussed. Several examples of such thematic functions do exist. For instance, vasopressin promotes water retention in the kidney, causes vasoconstriction, and stimulates water intake by acting in brain, all functions that relate to available fluid volume in the body and the circulatory system. Oxytocin (OT) stimulates uterus contractions during birth and myoepithelial

contractions in the mammary gland as a peripheral hormone, and it promotes emotional bondage as a neuropeptide, functions that can easily be summarized as being related to reproduction and social bonding. On the other hand, OT is also involved in descending projections from the hypothalamus to the hindbrain that modulate satiation signals, a function that cannot directly be related to reproduction or social bonding. Also, a "thematic" function can hardly be detected for several other neuropeptides: Neuropeptide Y, for instance, is anabolic, anxiolytic, and has been implicated in cell proliferation and differentiation. The cocaine and amphetamine-regulated transcript (CART) is involved in mediation of such diverse functions as pain and eating.

In general, evolutionary pressures likely take advantage of available compounds for novel functions; e.g., a peptide with one original function may over time develop novel functions related to that original "theme" as well as to divergent functions. Over time, this may lead to new compounds (e.g., ancestral insulin evolved into "modern" insulin and insulin-like growth factors) or simply apparently unrelated functions of the same compound. Another important point relates to the anatomical sphere of influence of the compound. For example, circulating hormones may be more likely to have a thematic function because receptors for it in diverse tissues are accessible from the circulation, whereas a thematic function may be less likely for a neuropeptide because the release and action of the peptide is confined to individual, isolated locations.

In short, while the answer to the question of "thematic functions" of signaling compounds is probably too complex to have been discussed comprehensively in the available time, there was consensus that a unified physiological or "thematic" function is certainly not a universal principle. Biological systems are considered to have evolved using whatever ligands and receptors are available to provide important signaling capacity, and the needs might well differ among systems. Biological systems are modular, with many interacting parts and levels, even within a single cell.

Nuclei in the nervous system were originally defined morphologically rather than functionally, a categorization that still remains the basis for the majority of standard animal brain atlases. It is clear, however, that the vast majority of brain nuclei contain diverse cell types that influence diverse physiological systems via diverse axonal projections. Thus, a neuron may synthesize numerous transmitters (peptides, biogenic amines), with different subsets released at different terminals or in the same terminal under different conditions and on a different time scale. As sophisticated techniques became available, and single cells could be phenotyped, the functional diversity of subsets of cells in numerous brain nuclei became apparent. That said there are also examples of nuclei or portions of nuclei in which there is a single, dedicated function. Generally, these are nuclei that are closely allied to sensory or motor functions. For example, this may be the case for autonomic motor nuclei in the hindbrain or for some sensory nuclei (e.g., sensory representation of inputs from the whiskers in the barrel cortex).

4.2. Peptide receptor function

The group discussed challenges associated with investigating the function of G-protein coupled receptors (GPCR) in the brain. GPCR are the largest family in the mammalian genome and represent the targets of many drugs. Therefore, given the relevance of this topic for the participants, we have included an extended discussion of it here.

GPCR function can easily be misinterpreted. This appears to be an underappreciated problem that primarily derives from two technical limitations. First, accurately locating GPCRs within brain cells; and second, from the techniques available to manipulate their function. Accurately locating GPCRs in the brain—particularly at the sub-cellular level—is not a trivial task. They are found post-synaptically on dendrites and neuronal soma and pre-synaptically on axon terminals where they often reside somewhat distally from the synaptic cleft. For the most part, GPCR ligands act as modulators rather than mediators of ionotropic neurotransmission. In addition to occurring on neurons, GPCRs are also expressed by glial, endothelial, epithelial, and ependymal cells, complicating how experimental manipulations must be interpreted.

Accurate GPCR localization is hampered by the lack of suitable probes, particularly high specificity antibodies. For example, commercially available GPCR antibodies are often poorly characterized, meaning that they may provide little useful information. As an alternative, GPCR location can be addressed by means of what are essentially proxy approaches. Two are commonly used: 1) appropriate gene promoters drive the expression of fluorescent markers in target cells; or 2) in situ hybridization (ISH) is used to locate GPCR encoding mRNAs. While both techniques have greatly advanced our knowledge about which specific cell types express GPCRs, neither provides information about the precise subcellular location of target GPCRs, nor how altering their function impacts a neural circuit after a manipulation. For example, it is not clear how the distribution of a GPCR gene promoter-driven GFP signal in a neuron relates to the specific location of the functioning transmembrane receptor protein; while in-situ-hybridization identifies mRNA, and not protein. This situation could be dramatically improved by developing antibodies that are much better targeted to the functionally active epitopes of GPCRs.

With regard to investigating GPCR function, tools are again less than ideal. Traditional pharmacology offers receptor sub-type specificity, but targeted delivery is not always well controlled. An alternative and ostensibly more targeted approach uses shRNA or other methods to knock down (KD) receptor gene expression. However, the way that results from some gene KD experiments appear to be interpreted raises the possibility that the location of the GPCR affected by the KD is not always carefully considered. It should be remembered that a manipulation that reduces the amount of a GPCR mRNA in a target brain area likely only affects receptor expression in neurons that have their cell bodies within the area of the injection. This is important because any presynaptic GPRCs found on afferent neurons projecting into the target area are unaffected by the KD; these are synthesized by distally located neuronal populations. The fact that GPCRs can be found on the axon terminals of target neurons also means that loss of function is unlikely to be confined just to the region containing its soma and dendrites. The efferent projections of these neurons will also lose their pre-synaptic GPCRs, and these may be some distance away. Interpreting the effects of mRNA KDs is therefore far from straightforward, and it is unhelpful that some studies appear to conflate the KD of GPRC mRNAs in neurons within a region with a reduction/loss of all cognate receptor proteins throughout that region, which probably doesn't occur because of presynaptic receptor distribution.

Discussions concluded that the combination of a lack of methods for accurate localization and of the site-specific compromise of function means that current methods still lack the specificity to address GPRC function in a sufficiently sophisticated manner.

4.3. Redundancy

Why does so much redundancy exist in some biological systems? As an example, why are there so many peptides and other eating-generated molecules that act to reduce meal size? The overwhelming response from the participants was that this is what it takes for the system to function optimally. While numerous peptides reduce

food intake as a collective, perhaps redundant, activity, each also has other unique features. The redundancy for some activities (e.g., ingestive behavior) makes the overall metabolic process more efficient and emphasizes how critical adequate energy is for the two principal biologic goals — survival and reproduction. For example, whether or not to eat and how much to eat depend on complex economics including prey/predator probabilities, the energy it takes to forage and obtain food, the amount of stored energy on hand, idiosyncratic factors such as stress or illness, etc. Therefore, the "appropriate" decision is the result of compromises or balances of competing goals (e.g., to acquire calories without becoming prey or expending more calories than are gained in the search for food). There may be a greater incidence of this redundancy in the neural processing of sensory signals or for life sustaining activities. We see it as a redundancy but it may be an artifact of our measuring a single variable at a time, using assays and measurements that have been standardized across laboratories in order to increase interpretive power. However, these may well miss or even obscure finer behavior details that are unique to a particular signaling pathway.

5. PERSPECTIVES/EPILOGUE

As alluded to in the beginning, science at large, as well as research in our field, is currently facing several serious problems: decreases in funding, bad public opinion/perception of research, questions concerning honesty of the actors, reproducibility and/or relevance of the data, etc. We as scientists need to be open to justified criticism from the outside. In particular, as some of these criticisms raise questions about the entire "operating system" of science as a whole. It is clear that doing nothing would be the worst strategy, because it would further discredit science and eventually result in "punishments" by funding organizations and the public. Thus, we need to find answers to the questions and solutions to the problems. But what are these answers and solutions? What is the best way forward and how should we proceed with respect to the various issues where action is needed?
Although the meeting, naturally, did not cover all of the critical issues, the discussions touched upon a broad range of topics that are important for the future of this field of research. Reflecting on the combined thoughts and thorough analysis of a large group of excellent scientists, the results of these discussions may suggest at least some possible ways to proceed. In this spirit, we hope that this summary of the major ideas of the meeting may help to promote this important discussion for our field.

CONFLICTS OF INTEREST

None of the authors declares any conflict of interest.

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