Minireview

Functional genomics: the search for novel neurotransmitters and neuropeptides

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Abstract Functional genomics can be defined as the search for the physiological role of a gene for which only its primary sequence is known. Most of the genes encoding proteins containing seven hydrophobic stretches code for G protein-coupled receptors (GPCRs). Although many of these have been shown to interact with known natural ligands, several bind ligands which have not been thus far isolated. These are the so-called orphan GPCRs. As an example of functional genomics, an 'orphan receptor strategy' has been developed to identify the natural ligands of orphan GPCRs. The application of this strategy is bound to revolutionize our understanding of the diversity of the primary messengers which modulate synaptic transmission. This review discusses the basic concepts and some of the particular problems associated with the orphan receptor strategy. The strategy's potential is exemplified by its successes which culminated in the discovery of the neuropeptides 'orphanin FQ/nociceptin' and 'orexins/hypocretins'. The steps that led to the characterization of these neuropeptides are discussed as are some of the further studies that have addressed the roles of these neuropeptides. To conclude, some of the implications of the application of the orphan receptor strategy are discussed.

Key words: Orphan G protein-coupled receptor; Neurotransmitter; Neuropeptide; Neurobiology; Precursor; Gene; Functional genomics

1. The orphan receptor strategy

1.1. The existence of orphan receptors

The hypothesis that transmitter receptors (neurotransmitters, neuropeptides, peptide hormones, eicosanoids, olfactory receptors) belong to supergene families [1] underlies the belief that homology screening approaches would lead to the identification of novel receptors. These approaches suffered from one obvious problem: how to find the pharmacological characteristics of the newly cloned receptors? By definition, the receptors cloned via these strategies would be in search of their pharmacological identity, their natural ligand. They indeed were all 'orphan' receptors, i.e. receptors in search of a function. The pursuit to unravel the identities of orphan receptors was thought to be a formidable, and to many unresolvable, task which would lead to unglamorous fishing expeditions (surprisingly, this proved not to be the case for the first 'orphan' G protein-coupled receptor (G-21) [2] which was soon demonstrated to be the 5-HT_{1A} receptor [3]).

The problem of the pharmacological identity of orphan receptors became more prominent when approaches specifically directed at the identification of G protein-coupled receptors (GPCRs) by homology screening were developed. The first one, based on low-stringency hybridization, led to the cloning of several orphan receptors [4], one of which was shown to be the dopamine D_2 receptor. The second, a PCR-based approach, also led to the cloning of several orphan receptors [5], some of which awaited patient searches to finally find their pharmacological identity. The application of these technologies resulted in two major advances.

First, they led to the cloning of most of the pharmacologically expected receptors and their, sometimes unexpected, subtypes. To date, cloned GPCRs exist for all the known neurotransmitters, neuropeptides and peptide hormones [6,7]. Second, they led to the identification of receptors which have kept their orphaned status until this day. These receptors must bind ligands which have not been thus far characterized, because inactive receptors should be evolutionarily discarded.

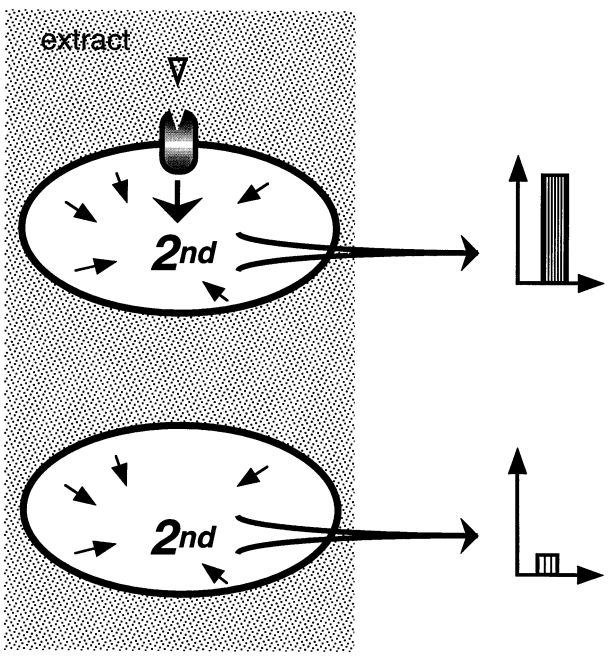

Fig. 1. Schematic representation of the orphan receptor strategy. A cell transfected with an orphan GPCR is exposed to a tissue extract containing the putative natural ligand (top). The ligand receptor interaction induces a second messenger response (large arrow). However, the complexity of the extract allows for additional molecules to induce responses (small arrows) making the total response (2nd) the sum of the orphan GPCR-induced and endogenous responses. The level of the endogenous responses can be determined in untransfected cells (bottom).

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This fact inspired enough confidence in a few researchers [8] to utilize orphan receptors as bait to isolate their natural ligands, which meant identifying novel transmitters (an approach reviewed as a 'neglected opportunity' [9]).

1.2. The strategy and its pitfalls

The concept that an orphan receptor could be used as bait to identify a novel transmitter is new in that it implies the use of a molecular clone but relies on the same path as the ones which led to the identification of neuropeptides using biological assays. The discovery of the opioid peptides [10] is an example of this. The principle of this concept is to transfect the cloned orphan receptor into a cell which consequently can be activated by the orphan receptor ligand. By exposing the transfected cell to a tissue extract containing the natural ligand of the orphan receptor, a change in intracellular second messengers will be induced and will serve as a parameter to monitor the orphan receptor ligand purification (Fig. 1).

This concept butts against two unknowns: the physical nature of the ligand and the type of second messenger response that it will generate. Both of these characteristics are unknown. GPCRs bind amino acids, biogenic amines, peptides, proteins, eicosanoids, lipids, odorants and others. There is not a single technique which will isolate all of these compounds in bioactive forms. Moreover, GPCRs couple to G proteins to modulate the activities of adenylyl cyclase, phospholipase C and A_2, channels, phosphodiesterase and possibly other second messenger effectors [11]. There are no rules ascertaining which second messenger will be modulated by an orphan receptor. Attempting to identify the natural ligand of an orphan receptor is short of facing a single equation with two unknowns (Fig. 2).

There are, however, structural features in orphan GPCRs which can be used to advantage in evaluating the nature of their ligands and the second messengers that they will activate. Analysis of the primary sequence of a GPCR will determine its relationship to known receptors. A significant relationship can help in evaluating the nature of the receptor's ligand and its activity. An orphan receptor which is related, even in a low degree, to a particular receptor family has a higher probability of sharing a ligand of the same physical nature and a coupling to similar G proteins. Although there is no certainty that similar receptors will bind ligands of similar nature and similar G protein couplings, it is a fair start for such a search.

The first obstacle met in identifying the ligand of orphan receptors is our inability to ensure that an orphan receptor transfected cell line does indeed express the receptor. Northern blot analysis can ascertain that a corresponding mRNA is expressed but is not a sufficient criterion to ensure the expression of the protein. Expressing tagged receptors could be a solution but one which opens the possibility that the tag may interfere with the ligand binding or the receptor coupling. A simpler, but more demanding, way to overcome this obstacle is to establish several lines of orphan receptor expressing cells and to carry them in parallel along the purification steps. These lines can be established in different cellular environments which further increases the chance of finding the orphan receptor induced second messenger system.

The chemical nature of the natural ligand is a major unknown in orphan receptor research. There exists no single purification technique which would allow for the isolation of all the physically different orphan receptor ligands. A choice has to be made from the start. Thus far, ligands of orphan receptors have been isolated which are peptides or lipids [12–16]. In every case, the primary sequence of the orphan receptor directed the choice of isolation procedure. Noteworthy, the identification of novel lipophilic ligands has thus far relied on testing a battery of synthetic compounds instead of extracting them from tissue extracts.

The second major unknown in the search for orphan receptor ligands is to find out which second messenger system the orphan receptor will induce. Here again, the primary sequence of the orphan receptor is a revealing feature. GPRCs can couple to different second messenger systems in different cellular environments [17]. It is expected that the G protein content of a cell is the predominant factor directing which second messenger systems will be induced by a GPCR. A GPCR induces at least one 'obligatory' second messenger pathway (its principal pathway) and, in most cases, several 'accessory' pathways, which are possibly less efficiently induced but which will be detectable in artificial environments [17]. If it is impossible a priori to ascertain the 'obligatory' induction pathway of an orphan receptor, one may express it in different cellular environments where its 'accessory' induction pathway will be better revealed. One may also modify the G protein content of

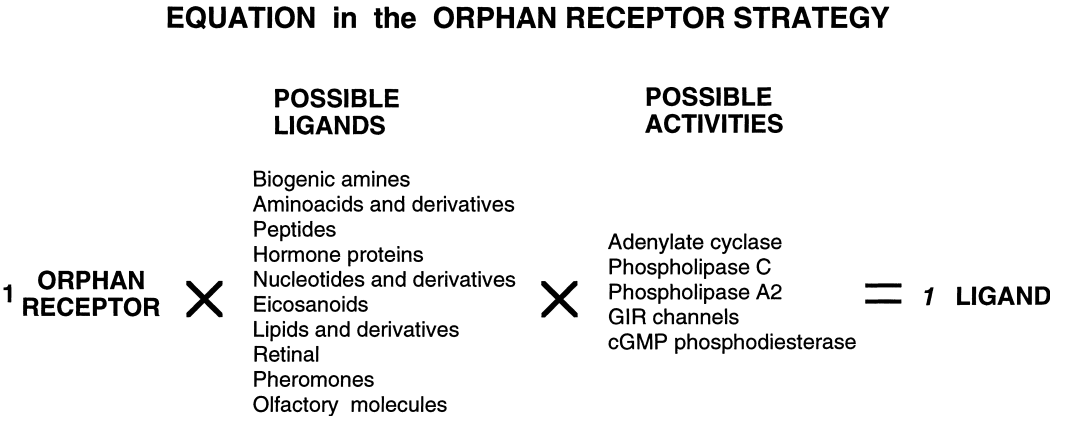

Fig. 2. The inherent dilemma of the orphan receptor strategy. The chemical nature of the orphan receptor ligand and the intracellular responses that it may induce are unknown. Theoretically, the number of permutations in this equation makes the search for the natural ligand of an orphan receptor an impossible task. However, as described in the text, this equation can be resolved.

a cell to direct an orphan GPCR to a particular pathway. Furthermore, considering that the vast majority of the GPCRs modulate partly either adenylyl cyclase activity or intracellular calcium release, one can expect that any orphan receptor will affect at least one of these second messenger systems if tested in a couple of different cellular environments. Alternatively, one may monitor the activity of all orphan GPCRs using GTP-γS binding. Recording G protein induction via this methodology is seemingly universal, but has not been applied thus far in orphan GPCR research.

2. Application of the orphan receptor strategy to the isolation of novel neuropeptides

2.1. Identification of OFQ/Noc and of OX/Hcrt

The first deliberate attempt at identifying the ligand of an orphan receptor took advantage of a particular receptor. A GPCR had been cloned which exhibited about 65% sequence identity with the three opioid receptors but which had been shown not to bind any of the natural or synthetic opioids or opiates [8,18,19]. Because of its similarities to the opioid receptors, it was assumed that this receptor might also bind a peptidergic ligand and share the same coupling mechanism to second messenger systems as that of the opioid receptors, i.e. inhibition of adenylyl cyclase activity.

Purification of the natural ligand of the opioid-like orphan receptor was achieved simultaneously in two different laboratories starting from either rat or porcine brain extracts [12,13]. Both approaches led to the identification of a 17 residue long peptide with the primary structure FGGFTGARKSARKLANQ. This peptide was named orphanin FQ (OFQ) or nociceptin (Noc). The striking features of OFQ are its similarities to the opioid peptides, most notably its amino-terminal tetrapeptide FGGF reminiscent of the canonical YGGF of the opioid peptides.

The second attempt at applying the orphan receptor strategy was more general. Over 50 different cells were established each expressing different orphan GPCRs. They were tested for their abilities to induce intracellular calcium release when subjected to peptide extracts prepared from hypothalami. One cell line did respond and led to the characterization of two peptides, the orexins (OXs) which are structurally related and amidated [15]. These peptides had been previously identified to be expressed in the hypothalamus and named hypocretins (Hcrts) [20].

2.2. Characterization of the orphan receptor ligands

For a ligand to be specific to its receptor, it must bind this receptor with saturable, displaceable kinetics and with high affinity. Extracts of organs, especially complex ones such as brain, contain numerous catabolic products which may bind to receptors without being specific ligands. OFQ/Noc and OX/Hcrt were shown to bind and activate their corresponding orphan receptors with constants in the low nanomolar range (K_d 0.1 and 20 nM and EC_{50} 1.05 and 30 nM, respectively) [13,15]. Although the question remains open whether these peptides are the sole ligands to their respective orphan receptor, these data demonstrated they are natural ligands of the orphan receptors.

Bioactive peptides are synthesized as part of larger polypeptides. Cloning the precursors encoding the newly isolated peptides can not only ascertain their classification as bioactive but may also indicate the existence of other co-encoded peptides. The OFQ/Noc and OX/Hcrt precursors were cloned [21,22,15]. Both precursors exhibit all the characteristics expected of a neuropeptide precursor, in particular a signal sequence necessary for their secretion and pairs of basic amino acid residues flanking the peptide sequences which constitute recognition sites for prohormone convertases. These data showed that OFQ/Noc and OX/Hcrt can act as bioactive peptides. They further demonstrated that the two OXs/Hcrts are encoded by a single precursor. In the case of OFQ/Noc, they raised the possibility that other bioactive peptides may be generated by the OFQ/Noc precursor [23]. The availability of the precursor cDNAs allowed for analyses of their site of synthesis and the demonstration that OFQ/Noc and OXs/Hcrts are synthesized in the CNS [20,24,25]. This fact supported their classification as neuropeptides.

The next step in characterizing orphan receptor ligands is to search for their physiological roles. Localization of the peptides by immunoreactivity and of precursors and receptors by in situ hybridization studies is important information toward this goal. Because of their detection in the lateral hypothalamus, OXs/Hcrts were expected to regulate feeding behavior and energy homeostasis. Indeed, OXs/Hcrts, when injected intracerebroventricularly (i.c.v.), stimulated food consumption in a dose-dependent manner [15]. The broad distribution of the OFQ/Noc system, on the other hand, suggested that it will regulate many behavioral responses. To date OFQ/Noc has been implicated in a dozen physiological responses. OFQ/Noc was first shown to modulate locomotion and to affect pain perception. The effects of OFQ/Noc on nociception have been intensely debated and have been reviewed [18,19]. Originally OFQ/Noc was reported to have a hyperalgesic activity, an effect later shown to result from a reversal of stress-induced analgesia [26]. However, depending on the doses and the route of administration, OFQ/Noc has also been reported to induce analgesia [27], and furthermore to induce allodynia in response to innocuous tactile stimuli [28]. Because of its similarity to the opioid peptides, OFQ/Noc was tested for its ability to produce reinforcement behavior [29] and was shown, in contrast to morphine, not to produce conditioned place preference or aversion. OFQ/Noc was further shown to produce an impairment of spatial learning [30], to produce marked changes in the renal excretion of water and sodium [31], to exhibit vasorelaxant properties [32], to induce hypotension and cardiac output [33,34] and to affect sexual behaviors by facilitating lordosis [35] and penile erection [36]. Similar to the Oxs/Hcrts, OFQ/Noc was reported to induce feeding in satiated rats [37,38]. Finally, OFQ/Noc has been shown to induce a profound anxiolytic response in mice and rats [39] in a manner similar to that produced by diazepam.

3. Conclusion: implications of the orphan receptor strategy

Because it permits the identification of the natural ligand of a putative receptor known only from its primary sequence, the orphan receptor strategy stands as an example of a successful attempt in functional genomics. This strategy, however, makes us face two questions. The first relates to the identity of the isolated ligand. A GPCR can bind different ligands at different affinities. The isolated ligand may interact with the receptor without being a truly bioactive molecule. For example, degradation products of abundant proteins may bind and

activate a receptor (as was the case of the isolation of OFQ/Noc [13]). Of course in the case of peptides, the cloning of their precursors is an important step in overcoming this problem. But ultimately the possibility that the isolated ligand is not a bioactive one remains until a physiological response is shown to be directly modulated by the ligand.

The second question associated with this strategy relates to the identification of the central physiological roles of the novel ligand. Many neurotransmitters and neuropeptides are implicated in numerous behavioral responses, for example, OFQ/Noc has already shown this tendency. How to define the principal role(s) of a novel ligand will be the central aim of future studies. This underscores the biggest obstacle, and perhaps the greatest outcome, of orphan receptor research: our lack of behavioral assays monitoring complex or 'higher' brain functions. It is hoped that the description of novel 'orphan' neuronal systems and their subsequent genetic manipulation (as already begun in the case of the OFQ/Noc system [40]) will help to develop assays describing novel behavioral responses. But in the end, it will be the understanding of the implication of the novel ligand system in the human organism which will need to be analyzed. This aim unfortunately will be demanding in time and effort, but the fact that GPCRs are established as the prominent class of therapeutic targets indicates that orphan GPCRs will be the object of drug discovery programs.

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References

[1] Hall, Z.A. (1987) Trends Neurol. Sci. 10, 99–100.
[2] Kobilka, B.K., Frielle, T., Collins, S., Yang-Feng, T., Kobilka, T.S., Francke, U., Lefkowitz, R.J. and Caron, M.G. (1987) Nature 329, 75–79.
[3] Fargin, A., Raymond, J.R., Lohse, M.J., Kobilka, B.K. and Caron, M.G. (1988) Nature 335, 358–360.
[4] Bunzow, J.R., Van Tol, H.H., Grandy, D.K., Albert, P., Salon, J., Christie, M., Machida, C.A., Neve, K.A. and Civelli, O. (1988) Nature 336, 783–787.
[5] Libert, F., Parmentier, M., Lefort, A., Dinsart, C., Van Sande, J., Maenhaut, C., Simon, M.J., Dumont, J.E. and Vassart, G. (1989) Science 244, 569–572.
[6] Horn, F., Weare, J., Beukers, M.W., Horsch, S., Bairoch, A., Chen, W., Edvardsen, O., Campagne, F. and Vriend, G. (1998) Nucleic Acids Res. 26, 275–279.
[7] Kolakowski, L.F. and Zhuang, J., http://www.gcrdb.uthscsa.edu.
[8] Civelli, O., Nothacker, H.P. and Reinscheid, R. (1998) Crit. Rev. Neurobiol. (in press).
[9] Stadel, J.M., Wilson, S. and Bergsma, D.J. (1997) Trends Pharmacol. Sci. 18, 430–437.
[10] Hughes, J., Smith, T.H., Kosteritz, J.W., Fothergill, L.A., Morgan, B.A. and Morris, H.R. (1995) Nature 358, 577–579.
[11] Ross, E.M. (1992) in: An Introduction to Molecular Neurobiology (Hall, Z.W., Ed.), pp. 181–206, Sinauer Associates, Sunderland, MA.
[12] Meunier, J.C., Mollereau, C., Toll, L., Suaudeau, C., Moisand, C., Alvinerie, P., Butour, J.L., Guillemot, J.C., Ferrara, P. and Monsarrat, B. et al. (1995) Nature 377, 532–535.
[13] Reinscheid, R.K., Nothacker, H.P., Bourson, A., Ardati, A., Henningsen, R.A., Bunzow, J.R., Grandy, D.K., Langen, H., Monsma Jr., F.J. and Civelli, O. (1995) Science 270, 792–794.
[14] Okuda-Ashitaka, E., Tachibana, S., Houtani, T., Minami, T., Masu, Y., Nishi, M., Takeshima, H., Sugimoto, T. and Ito, S. (1996) Mol. Brain Res. 43, 96–104.
[15] Sakurai, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P. and Wilson, S. et al. (1998) Cell 92, 573–585.
[16] Lee, M.J., Van Brocklyn, J.R., Thangada, S., Liu, C.H., Hand, A.R., Menzeleev, R., Spiegel, S. and Hla, T. (1998) Science 279, 1552–1555.
[17] Civelli, O., Bunzow, J.R. and Grandy, D.K. (1993) Annu. Rev. Pharmacol. Toxicol. 32, 281–307.
[18] Henderson, G. and McKnight, A.T. (1997) Trends Pharmacol. Sci. 18, 293–300.
[19] Meunier, J.C. (1997) Eur. J. Pharmacol. 340, 1–15.
[20] de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L., Gautvik, V.T. and Bartlett II, F.S. et al. (1998) Proc. Natl. Acad. Sci. USA 95, 322–327.
[21] Mollereau, C., Simons, M.J., Soularue, P., Liners, F., Vassart, G., Meunier, J.C. and Parmentier, M. (1996) Proc. Natl. Acad. Sci. USA 93, 8666–8670.
[22] Nothacker, H.P., Reinscheid, R.K., Mansour, A., Henningsen, R.A., Ardati, A., Monsma Jr., F.J., Watson, S.J. and Civelli, O. (1996) Proc. Natl. Acad. Sci. USA 93, 8677–8682.
[23] Okuda-Ashitaka, E., Minami, T., Tachibana, S., Yoshihara, Y., Nishiuchi, Y., Kimura, T. and Ito, S. (1996) Nature 392, 286–289.
[24] Anton, B., Fein, J., To, T., Li, X., Silberstein, L. and Evans, C.J. (1996) J. Comp. Neurol. 368, 229–251.
[25] Neal, C.R., Mansour, A., Nothacker, H.P., Reinscheid, R.K., Civelli, O. and Watson, S.J. (1998) (submitted).
[26] Mogil, J.S., Grisel, J.E., Reinscheid, R.K., Civelli, O., Belknap, J.K. and Grandy, D.K. (1996) Neuroscience 75, 333–377.
[27] Rossi, G.C., Leventhal, L. and Pasternak, G.W. (1996) Eur. J. Pharmacol. 311, R7–R8.
[28] Hara, N., Minami, T., Okuda-Ashitaka, E., Sugimoto, T., Sakai, M., Onaka, M., Mori, H., Imanishi, T., Shingu, K. and Ito, S. (1997) Br. J. Pharmacol. 121, 401–408.
[29] Devine, D.P., Reinscheid, R.K., Monsma Jr., F.J., Civelli, O. and Akil, H. (1996) Brain Res. 727, 225–229.
[30] Sandin, J., Georgieva, J., Schott, P.A., Ogren, S.O. and Terenius, L. (1997) Eur. J. Neurosci. 9, 194–197.
[31] Kapusta, D.R., Sezen, S.F., Chang, J.K., Lippton, H. and Kenigs, V.A. (1997) Life Sci. 60, PL15–PL21.
[32] Gumusel, B., Hao, Q., Hyman, A., Chang, J.K., Kapusta, D.R. and Lipton, H. (1997) Life Sci. 60, PL141–PL145.
[33] Champion, H.C. and Kadowitz, P.J. (1997) Biochem. Biophys. Res. Commun. 234, 309–312.
[34] Champion, H.C. and Kadowitz, P.J. (1997) Life Sci. 60, PL241–PL245.
[35] Sinchak, K., Hendricks, D.G., Baroudi, R. and Micevych, P.E. (1997) NeuroReport 8, 3857–3860.
[36] Champion, H.C., Wang, R., Hellstrom, W.J. and Kadowitz, P.J. (1997) Am. J. Physiol. 273, (Pt. 1) E214–E219.
[37] Pomonis, J.D., Billington, C.J. and Levine, A.S. (1996) NeuroReport 8, 369–371.
[38] Stratford, T.R., Holahan, M.R. and Kelley, A.E. (1997) NeuroReport 8, 423–426.
[39] Jenck, F., Moreau, J.L., Martin, J.R., Kilpatrick, G.J., Reinscheid, R.K., Monsma Jr., F.J., Nothacker, H.P. and Civelli, O. (1997) Proc. Natl. Acad. Sci. USA 94, 14854–14858.
[40] Nishi, M., Houtani, T., Noda, Y., Mamiya, T., Sato, K., Doi, T., Kuno, J., Takeshima, H., Nukada, T. and Nabeshima, T. et al. (1997) EMBO J. 16, 1858–1864.