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Morphinome – proteome of the nervous system after morphine treatment

Review Article

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Summary. Proteome is a natural consequence of the post-genome era when the HUGO project (Human Genome Organization) has almost been completed. Here, a specifically aimed proteome in drug dependence – morphinome, is described, including tasks, strategies and pitfalls of the methodology.

Keywords: Morphine – Dependence – Cell culture – Brain – Nervous system – Proteome – Proteins – Analysis – Peptide map – Sequence – Mass spectrometry – Electrophoresis

Introduction

The first protein studies, which we can refer to as proteomics began in 1975 with the introduction of the 2-D gels by O'Farrell, Klose and Scheele. Klose used this technique to analyze mouse tissues (Klose, 1975), Scheele performed similar work with guinea pig exocrine pancreatic proteins (Scheele, 1975) and O'Farrell introduced at the same time high-resolution two-dimensional electrophoresis (O'Farrell, 1975). Although a vast number of proteins could be separated from a complex mixture and visualized, they still could not be identified. Whereas the development of 2-DE was a major step forward, the science of proteomics had to wait until the displayed proteins could be analyzed. The first major breakthrough was development of biological mass spectrometry with its Fast Atom Bombardment (FAB) ionization, allowing, for the first time, analysis of biomolecules directly in solution (Barber et al., 1981; Williams et al., 1981). Further developments of electrospray ionization (ESI) (Fenn et al., 1990) and matrix-assisted laser desorption/ionization (MALDI) (Hillenkamp et al., 1990; Tanaka et al., 1988) allowed for a sensitive and unambiguous identification of peptides and proteins including their amino acid sequences (Yates, 1998; Davidsson et al., 2003; Standing, 2003).

Addiction is a very complex process, which exerts its influence on molecular mechanisms, and is associated with psychological changes as well as with the changes in physiology of the brain and its specific structures, involved in this phenomenon. Among them are: ventral tegmental area (VTA) in the mid-brain (Koeltzow et al., 2003), nucleus accumbens (Nac) (Deadwyler, 2004) at the base of striatum, and prefrontal cortex (PFC). They together create a circuit, which is called the reward system. An excellent review on drug dependence was recently published by Kreek et al. (Kreek et al., 2002). But still, because of its complexity, many aspects of addiction remain unclear (Elkashef et al., 2003) and demand further investigation.

What is obvious, such complex phenomena can not occur without modifications in protein patterns in these structures, where addiction takes its origin, so during past few years many efforts were done to find protein or peptide markers of addiction.

It should be pointed out that many important classes of proteins, which could be involved in addiction and

serve as drug targets, such as transcription factors, protein kinases, and regulatory proteins, are present at low or very low quantities. These low levels of molecules will not be observed during analysis of crude cell lysates or tissue extracts, without pre-purification and pre-concentration steps. Therefore, dealing with a global phenotype is not a simple task and new methodologies must be developed and applied for subproteome identification (Lubec et al., 2003; Beranova-Giorgianni, 2003).

Here, we would like to introduce a new term for a specific proteome, which is focused on morphine dependence – ''morphinome'' to emphasize the complexity of the entire strategy and to reveal specific molecular mechanisms underlying drug dependence.

Changes associated with proteins during addiction – what we know today

Surprisingly, the search for the key words: ''proteomics AND morphine'' or ''proteomics AND dependence'' in the MEDLINE database, does not reveal any publications in this area. There are thousands of papers describing the influence of drugs and alcohol on particular protein, neuropeptide or behavior but analysis of proteome in drug dependence remains an open page.

At the beginning, investigations were focused on the family of endogenous opioid peptides: endorphins, enkephalins and dynorphins (Herz, 1993), which exert their influence on the brain via opioid receptors. Since morphine, the widest known addictive substance, acts via the same way, i.e. via μ-receptors, the conclusion was that these substances might be involved in processes connected to addiction. Endogenous opioid system seems to play a very important role in the entire process of drug abuse and withdrawal. During initial phase of dependence, endogenous opioids might mediate the reinforcing action of drugs. For example, animals are less sensitive to the reinforcing effect of cocaine after naltrexone pretreatment during initial phase of dependence, which confirms modulatory nature of endogenous opioid receptors during this phase (De Vry et al., 1989).

A decline in beta-endorphin level is observed in the anterior part of the limbic system in animals self-injecting heroin or cocaine before the session of daily drug intake which suggest its possible role in the dynamics of daily drug intake during the maintenance phase of addiction. (Sweep et al., 1988). Beta-endorphin in the limbic structures is also considered as neurochemical correlate of psychic dependence on drugs, since it level is similarly changed during heroin (which induces psychic and physical dependence) and cocaine (psychic dependence) addiction (Sweep et al., 1989; Gerrits et al., 2003).

Changes in levels of particular proteins and peptides are likely to be caused by changes in enzymatic activity of proteolytic enzymes. As an example, morphine exerts its influence on endopeptidases acting on dynorphin peptides, and causes increase in the conversion of dynorphin B (which is selective for kappa-opioid receptors) to Leu-enkephalin-Arg^6 (selective for delta-opioid receptors) (Vlaskovska, 1997). Chronic morphine treatment also increased the activity of endopeptidases, responsible for biotransformation of nociceptin/orphanin FQ (also endogenous opioid) into N/OFQ1-9 and N/OFQ1-13 fragments) (Vlaskovska et al., 1998).

Enzymatic activity very often depends on their phosphorylation state. Cyclic AMP response element-binding protein (CREB) is a transcription factor that mediates some of the effects of the cyclic AMP system on gene expression. Acute morphine administration decreases the state of phosphorylation of this protein, whereas chronic morphine administration attenuated this effect. During opiate withdrawal, the phosphorylation of CREB increases. Probably, in this way, opiates can produce changes in gene expression that lead to addiction (Guitart et al., 1992).

Chronic morphine treatment inhibits phosphorylation of SNAP-25 by inhibition of PKC activity, and leads to a down-regulation of SNARE complex formation, which is essential for vesicle release. This mechanism could be responsible for the alteration of exocytotic process and neural plasticity during opiate abuse (Xu et al., 2004).

Furthermore, changes in opioid receptor levels in different brain origin during different stage of drug abuse, could be observed (Fabian et al., 2002; Fan et al., 2003).

Appropriate models for drug dependence

Studies on the central nervous system in humans are technically difficult because of the limited availability of the tissue. Moreover, any ''model'' studies on humans are perceived as highly unethical. Therefore, the only source of information is cerebrospinal fluid, which, to certain extent, reflects dynamic changes in the nervous system due to its direct contact with the brain tissue. Another possibility is a tissue obtained post-mortem, however, optimization of the entire procedure can be difficult due to the law restrictions, time between death and tissue sampling and preparation, temperature control, etc. (Yang et al., 2004). Such studies are more often used for investigations of neurodegenerative disorders (Love, 2004; Verwer et al., 2003).

Cerebrospinal fluid was an object of extensive studies in pain (Harajiri et al., 1992), alcohol- (Silberring, 1994), and drug dependence but research was mainly focused on changes in neuropeptides and neuropeptide-degrading/converting peptidases (Persson et al., 1995; Nyberg et al., 1986). Recently, several papers were published, revealing proteinergic profiles in this fluid (Yuan et al., 2002; Silberring et al., 1989; Wetterhall et al., 2002; Ramstrom et al., 2003).

Other body fluids such as blood plasma, urine and saliva can also be of limited interest. From the clinical point of view, blood plasma would be the best source to study. But here are some technical limitations because of the presence of several abundant proteins (e.g. albumin or proteolytic enzymes). Those proteins present at high concentrations, can obscure the entire analysis and presently existing methodologies (e.g. immunoaffinity) to obtain albumin-depleted plasma are not very efficient and still under development (Rose et al., 2004). Analysis of urine is another alternative and this fluid has been extensively used in drug dependence but rather for the search for marker in drug abuse (Cone et al., 2003). Quantitative protein profiling in the urine requires its controlled collection over 24 hours, followed by concentration and further handling, which is a time consuming procedure. Besides, there is no strong evidence in literature that urine can reflect dynamic changes in the nervous system. This approach is, instead, commonly used to diagnose problems associated with e.g. kidney failure (Anderson et al., 2004) and for doping control (Hatton et al., 1987). Rapid accumulation of drugs and drug metabolites in saliva can also be an easy way of testing individuals for drug abuse (Maurer, 2004) but not for phenotyping changes in the brain.

Can we rely on animal experiments as model(s) of addiction?

As mentioned above, studies on humans are difficult or even impossible and to gain knowledge on the molecular mechanisms of drug addiction, scientists must apply either animal-, or cellular models. Thus, the following question appears: how close to reality those models are? Luckily, there are some factors that allow us to rely on animal experiments.

Human and rat opioid receptor systems, which are essential for morphine action in the brain, are similar, both in receptors sequences and their mechanism(s) of action in the cell (they both belong to the GPCR superfamily). Differences between them are not quite significant and are as follows: mu-human receptor is characterized by slightly higher affinity to morphine and methadone than in rats, despite the fact that these receptors are 95% identical at the amino acid level (Raynor et al., 1995). The sequence of human δ-receptor is 94% similar to the sequence of mouse δ-receptor, and it shows affinity to δ-ligands in transfected COS-1 cells (Simonin et al., 1994; Mestek et al., 1995). The human κ-receptor shows 93% sequence similarity to mouse- and rat κ-receptor and shows affinity to ligands characteristic for it (Zhu et al., 1995).

Techniques associated with neuroimaging as positron emission tomography (PET) and functional magnetic resonance (fMRI) allowed to examine the reward circuit in human brain during development of addiction (Leyton et al., 2002; Vollm et al., 2004). These experiments revealed structures connected with addiction in human brain and linked their activity to human behavior. These data confirmed observations performed on rodents and showed similarity between their circuits and the human's, thus creating the connection between animals used in laboratory experiments and addicted people (Volkow et al., 2003).

It should also be emphasized that only morphine gives a very well-defined model of addiction and withdrawal in mice and rats. Morphine treatment is well described and withdrawal signs are clear (e.g. commonly accepted wet-dog shakes). Other drugs, such as cocaine or amphetamine produce less significant, but still measurable, behavioral changes.

In this minireview, we will focus on the cells and brain tissue model of drug addiction due to some of their advantages. We must stress here that the cell model approach can only be considered as complementary research.

Cell culture and the whole tissue approach – between simplicity and reality

Advantages and disadvantages of cell cultures

Technologies associated with cell cultures make individual living cells accessible. Their differentiation and response to different stimuli could be observed and examined in the absence of the complexity of the entire organism. This is the first and main advantage of using such a model. Next, there is a possibility to obtain homogenous cultures of the cells by passaging. Furthermore, for each subculture, every replicate sample will be virtually identical, therefore examination of the influences induced by some stimuli and comparison with the

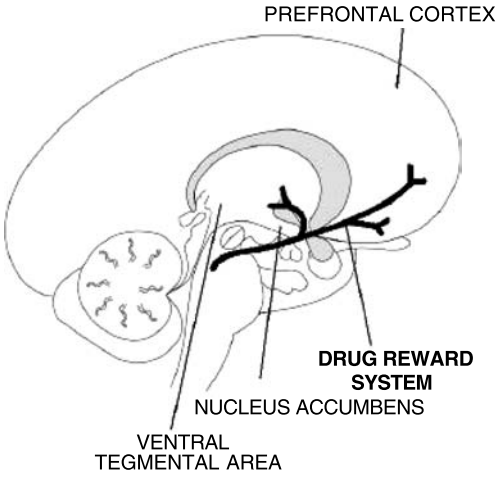

Fig. 1. The brain's drug reward system

same control culture, will be highly relevant and trustworthy (Suder et al., 2003; Vlaskovska et al., 1997a, b).

Reagents to which cells may be exposed could be administered directly at a defined concentration, which is one of the major drawbacks during *in vivo* experiments. Also, this technique ensures for significant control of the environment of the cell. The entire model can be well controlled and, thus, observation of dynamic changes in protein fingerprints over a time or after addition is possible and much easier than in the whole tissue (Hou et al., 1996).

In proteomics research simplicity, which is ensured by the cell culture model, is the greatest advantage. It is assumed that in a single cell about 1×10^6 proteins could be found, not to mention the number in the entire organ. By limiting the complexity of the system, one could help to obtain better results in searching for changes in protein expression or in revealing the entire proteomics pattern specific for a chosen tissue.

Unfortunately, this kind of approach has also its limitations and disadvantages. Culture techniques must be carried out under strict aseptic conditions, to avoid contamination by, bacteria for example. There can also be some differences in cell behavior between cultured cells and their counterparts *in vivo* and often discoveries done on this kind of models demand further confirmation (Freshney, 1990).

Whole tissue approach

Simplicity of the cell culture model is its main advantage. Such model, however, may not reflect real processes, which exist in the whole organism. To overcome this obstacle, specific tissue(s) should be examined. In case of proteomics research after morphine administration, brain structures that are associated with these phenomena are of main interest.

In this case, complexity of the whole system is maintained, but the proteins content, their amount and diversity is extended. This may cause further problems in their analysis. In particular, low abundance proteins could be undetectable in this case and prefractionation and enrichment procedures might be nessecary during preparation of the samples.

Strategies in morphinome identification

The beginning

In case of the cell culture approach, the first step of the experiment is to select the target cells (neurons or glial cells). The cells should be isolated from the organism and their culture must be established. The phenomenon of morphine dependence is mimicked in this model by addition of the appropriate amount of drug into the cell culture medium. One of the drawbacks is that nervous cells can not be kept in culture indefinitely.

In case of rats or mice, morphine dependence is usually induced by a subcutaneous implantation of pellets containing morphine, which imitates continuous administration of drug. After a certain time animals are decapitated and their brains isolated for further experiments.

Sample preparation

Sample preparation is always a crucial step as this might influence the entire procedure for protein identification.

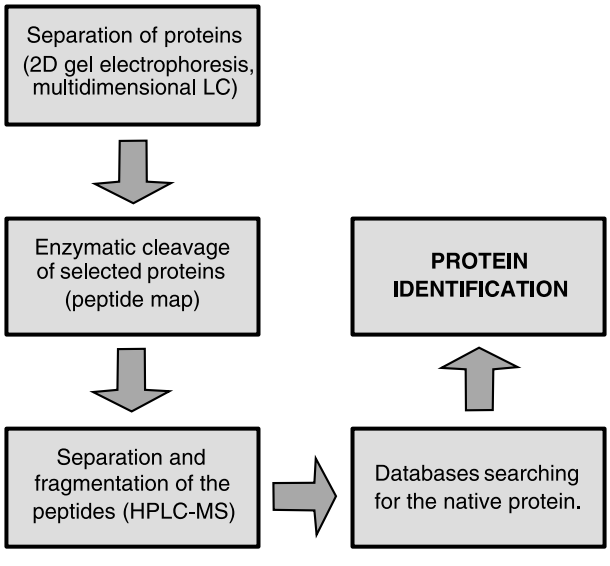

Fig. 2. Scheme of proceeding with the sample

Cell cultures are relatively easy to handle during this part of experiment. After time of establishing ''addiction'', the cells should be washed, to purify them from the culture media and homogenized under the proper conditions. Those conditions (buffers, pH, additives, etc.) should be carefully selected to maintain compatibility with further methodologies. Cells do not contain neither high amounts of abundant proteins (in relation to e.g. body fluids), nor lipids that are present in the nervous tissue so they do not need any kind of additional procedures to get rid of those ''impurities''.

The sample from the brain tissue is much more difficult to prepare. First of all, it demands great experience, because removal of the intact brain tissue should be as rapid as possible. It is well known that taking out the rat spinal cord requires less than one minute to be completed, if we need a tissue to study e.g. release of neuropeptides. The longer preparation time – the more artifactual results will occur (Przewlocki et al., 1986). Next difficulty results from the fact that this kind of sample usually requires additional steps associated with the removal of lipids and abundant proteins which complicates the entire procedure.

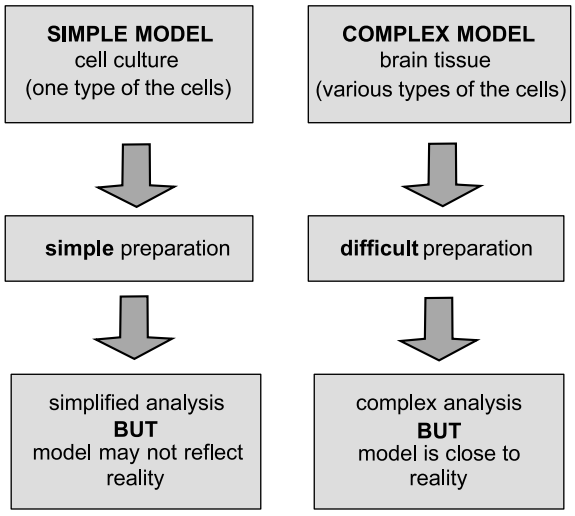

Fig. 3. The cell culture and the brain tissue – advantages and disadvantages

Scheme of proceeding with the sample

After appropriate preparation of the sample, further procedure is similar both for proteins obtained from the cell cultures and for those from the brain tissues. Proteins from ''control'' and ''addicted'' samples are separated by 2D gel electrophoresis and protein patterns characteristic for both of them are compared. Proteins that are found to be significant i.e. are characteristical for one sample, are chosen for further experiments.

During experiments, these proteins are cleaved, peptides obtained in this way are separated using liquid chromatography and molecular masses of those peptides are analysed by mass spectrometry. At the end, their amino acid sequence is revealed during MS/MS experiments.

Bioinformatics tools

Bioinformatic tools are essential at the end of the whole experiment. They allow for use the sequence of amino acid to reveal the examined protein identity (Yu et al., 2004). But the software and databases freely available on the Internet are, so far, the weakest point in proteomics strategy. Many efforts need to be done before these tools will become fully reliable. At present, it is even difficult to analyze a mixture of two peptide maps derived from proteins, coexisting in one gel spot. This fact implicates a very high resolution of the separation procedures.

Application of bioinformatics tools commonly available on the net in morphinome research is similar to other approaches. The major challenge is to create specific databases describing separate brain structures for particular species. Presently existing entries do not differentiate information obtained for selected tissues and the idea is to make new data bases that can be assembled as ''bricks'', depending on the ongoing project.

Conclusions

As it was shown above, in case of morphinomics there is no ideal approach. Cell cultures, as well as brain tissue models have their advantages and disadvantages, and scientists must be very careful while choosing between them. Also using computers and databases may not be free from unexpected difficulties and artifacts. Here, as a forewarning, we can include citation by Lubec and Pollak, recently published in ''Amino Acids'' (Lubec et al., 2004): ''Identification of pitfalls in proteomics is mandatory and the many newcomers in the booming proteomic area have to be trained to prevent overload literature with identifications just based upon ''highest score'' printed out from instruments.''

So far, drug dependence studies are still mostly performed by pharmacologists, and this is an important area of the research. However, knowledge on the molecular mechanisms involved in drug dependence is crucial for the design of new therapies. The important challenge in the next years will be investigation of the dynamic protein

expression and protein–protein interactions that are probably the key factors in understanding processes in the brain. These ambitious goals will certainly require involvement of a large number of different disciplines such as molecular biology, pharmacology, biochemistry, combinatorial chemistry, materials science, and bioinformatics. Assuming that a human being contains ca. 700.000 proteins, including those posttranslationally modified, and that those proteins interact with other molecules in a very subtle network(s), it is clear that we are very far from revealing true mechanisms underlying not only drug dependence but also basic processes that occur within the cell.

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