Report on the allegation of “likely suspected research misconduct” in the paper Ultrastructural localization of the alpha4-subunit of the neuronal acetylcholine nicotinic receptor in the rat substantia nigra. Journal of Neuroscience (1999) 19 6475-6487 by M M Arroyo-Jimenez , J P Bourgeois , L M Marubio , A M Le Sourd , O P Ottersen , E Rinvik , A Fairén , J P Changeux.

The aim of the research was to immunolocalize the alpha 4-subunit of the Neuronal Acetylcholine Nicotinic Receptor (nAChR) at the optical and ultrastructural level in the Rat Substantia Nigra.

The work published by Arroyo-Jimenez et al results from the cooperation of 8 authors from 4 different laboratories. It is a typical attempt to develop cooperation between laboratories with different and complementary scientific and technological competences. Jean-Pierre Bourgeois, from the Pasteur Institute, went to Oslo for about two weeks to carry out the immunogold labeling observations by electron microscopy following a method originally developed in his laboratory in Oslo and published under fig 9 -11 of Arroyo-Jimenez et al paper under the supervision of Ole Petter Ottersen.

PubPeer made the following comment two years ago:
This paper intends to localize a neurotransmitter receptor subunit in the brain of rodent. The localization is done by immunocytochemistry. The specificity of the antibody is shown on figure 1, where the first two lanes show the Ab on brain and lung, while the last two lanes show the blot on brain and lung after pre-absorption of the Ab with the peptide used to produce it. Except all three negative lanes are the same.

First of, PubPeer states, *The specificity of the antibody is shown on figure 1.* This statement is not entirely correct since the specificity of the antibody has been shown by two independent approaches as illustrated in **figure 1 and 2** (see below).

Second, figure 1 shows the “analysis of the alpha4 subunit antibody specificity by immunoblot of total homogenates of rat brain and lung” (left two lanes) and after “pre-adsorption by an alpha 4 derived synthetic peptide” corresponding to a portion of the intracellular domain of the rat nAChR alpha 4-subunit against which the polyclonal antibody (Santa Cruz Biotechnology, catalog #1772) was raised in the goat. The figure published in the printed version of J Neuroscience shows only one band in the left lane with an apparent molecular weight of ca 70kDa. This value is within the range where immune-purified alpha4-subunit protein as has been found by other authors (Whiting et al 1987).

Nevertheless, even if the three control lanes in figure 1 appear negative as expected for a specific antibody, PubPeer stated that *all three negative lanes are the same.* This comment has led to the allegation of “likely suspected research misconduct”.

This issue has been examined by the Comité de Déontologie from the Pasteur Institute. Using the software "IMAGE J" the analysis confirmed the nearly identity of the three control figures in the published picture.
As a first step in the attempt to understand this unexpected observation, the authors were asked to present, if available, the 20 years old raw data which were at the origin of figure 1. Dr. Maria Arroyo-Jimenez who carried out the experiment in the Pasteur laboratory was able to go back to the original notebooks she had preserved and to communicate a copy of the original photographs of the gels. Despite the rather low quality of the photograph, it is clear that the test lane shows exactly the same band at ca 70kDa which is **not** present the three control lanes, all this being consistent with the published figure 1 and most of all with the specificity of the antibody. Yet, the three control lanes show multiple faint bands differing from one lane to the other and corresponding to the background observed in such experiments. These raw data are thus consistent with the standard specificity test. The issue is then why such background micropatterns do not show up in the published figure?
Dr. Maria Arroyo-Jimenez in her remembering mentions " we cut the original film to do the final figure" and "I remember we adjusted the grayscale using Photoshop" (practices which were accepted at the time but no today). Thus a possible explanation is that after extensive reduction of the background, for an unknown reason, the three lanes appear the same. Nevertheless it cannot either exclude that the same lane has been accidentaly used.
In any case, whatever the final explanation of the published image, the evidence for the antibody specificity shown in figure 1 is consistent with the actual raw data.

This statement is further strengthened by the results summarized in figure 2. This second control was done independently, with the same antibody, on Human Embryonic Kidney cells (HEK-293) transfected, *in vitro*, with cDNAs combining diverse subunits of the nAChR: the rat $\alpha 3\alpha 4\beta 4$, or the human $\alpha 4$ &$\beta 2$, or a chimeric $\alpha 7$. Figure 2 unambiguously shows that immunolabeling was positive in membranes of cells transfected with either $\alpha 4\beta 4$ rat cDNAs or human a$4\beta 2$ cDNAs but *not* with chimeric-$\alpha 7$ or $\alpha 3\beta 4$, or in non-transfected cells. This experiment performed by one of the coauthors, *Lisa M Marubio,* then a postdoc student in the Pasteur laboratory, unambiguously shows the specificity of the antibody against the alpha subunit.

Last, it is important to note that a similar ultrastructural localization was independently found by immunogold labeling in Oslo and by immunoperoxidase labeling (in Pasteur).
In conclusion, one can assess that all the data presented in the paper on the ultrastructural localization of the alpha4-subunit of the neuronal acetylcholine nicotinic receptor in the rat substantia nigra by Arroyo-Jimenez et al 1999 are coherent with each other and consistent with the specificity of the antibodies available at that time.

Moreover, the Arroyo-Jimenez et al 1999 paper has been positively quoted by at least 19 articles in the past 20 years, without any negative or controversial statement.

In our opinion, the analysis of the data does not support the allegation of scientific misconduct.

François Rougeon

Chairman of the CVDC