Neuroscience and Biobehavioral Reviews 33 (2009) 33–47

Contents lists available at ScienceDirect

Neuroscience and Biobehavioral Reviews

journal homepage: www.elsevier.com/locate/neubiorev

Review

The 3R principle and the use of non-human primates in the study of neurodegenerative diseases: The case of Parkinson's disease

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ARTICLE INFO

Article history:
Received 23 July 2007
Received in revised form 11 August 2008
Accepted 11 August 2008

Keywords:
3R principle
Animal experimentation
Animal welfare
Bioethics
Non-human primates
Parkinson's disease

ABSTRACT

The aim of this paper is to offer an ethical perspective on the use of non-human primates in neurobiological studies, using the Parkinson's disease (PD) as an important case study. We refer, as theoretical framework, to the 3R principle, originally proposed by Russell and Burch [Russell, W.M.S., Burch, R.L., 1959. The Principles of Humane Experimental Technique. Universities Federation for Animal Welfare Wheathampstead, England (reprinted in 1992)]. Then, the use of non-human primates in the study of PD will be discussed in relation to the concepts of Replacement, Reduction, and Refinement. Replacement and Reduction result to be the more problematic concept to be applied, whereas Refinement offers relatively more opportunities of improvement. However, although in some cases the 3R principle shows its applicative limits, its value, as conceptual and inspirational tool remains extremely valuable. It suggests to the researchers a series of questions, both theoretical and methodological, which can have the results of improving the quality of life on the experimental models, the quality of the scientific data, and the public perception from the non-scientist community.

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doi:10.1016/j.neubiorev.2008.08.006

1. Introduction

The aim of this review is to offer an ethical point of view on the use of non-human primates in neurobiological studies. We have chosen the study of Parkinson's disease (PD) as a case study, and our methodological framework will be provided by the 3R principle originally proposed by Russell and Burch (1959).

In general, we feel that the experimental work carried out on non-human primates to study both the development and the possible therapies for PD, is a powerful and appropriate case-study to inspire fruitful discussion on the use of animals as models in biomedical research, also in the light of recent trends in neuroscience, where bioethical aspects have been gaining in consideration and importance. Although non-human primates represent a very little percentage of the total animals utilised (for example, 0.01% in 2005 in Great Britain) (Home Office, 2005), they occupy a special place in the discussion on the pro's and cons of animal experimentation. One of the reasons is that, due to their phylogenetical closeness with humans, it is thought that non-human primates can experience a similar level and kind of sufferance experienced by humans (see, for example, Cavalieri and Singer, 1993; King and Landau, 2003). As a matter of fact, it is very difficult to really know how and how much a non-human animal is suffering. This issue has been the object of analysis of some recent moral philosophers (see, for example, Armstrong and Botzler, 2003). Since the declaration of Jeremy Bentham (1748–1832) in favour of extending the principle of equality to non-human animals, many theories have been developed to face the central question of the moral status of animals. We can offer here a definition of moral status: individual A has a moral status if and only A's interests have some moral weight, independently of their effects on other beings who have moral status (De Grazia, 2003).

In fact, the judgement on the morality of experimentation on animals is directly dependent from the way we consider animals ought to be treated. And the way animals ought to be treated is deduced from the moral status we assign them. As a start, then, it must be answered the question whether animals can have moral value. We can say that, in practice, often animals are treated with moral respect. At least some animals (most of our pets) are already part of our moral community and have already moral status. If we look at our moral practice, it seems to be the capacity to suffer and experience pleasure and happiness to be the fundamental criterion to treat people with moral respect. The possibility to apply this reasoning to animals can be a useful contextual criteria to be used. Therefore, some authors suggest giving the animals the benefit of doubt (see, for example, Bekoff, 2002).

This idea is also supported by the ethical policy on the use of animals adopted by research institutions and scientific societies. For example, the National Institutes of Health Public Health Service Policy on Human Care and Use of Laboratory Animals, adopted by the Society for Neuroscience, argues that researchers should consider the fact that procedures that cause pain in humans will likely to cause the same pain in non-humans, unless the contrary is proved (National Institutes of Health, 2002). To consider in similar manner the level of sufferance potentially experienced by non-human primates to the one experienced by humans, make the use of monkeys in biomedical experimentation particularly relevant from an ethical point of view, although from a rather anthropocentric point of view.

If we adopt the "benefit of the doubt" approach to the level of sufferance experienced by PD monkeys, how should we consider the use of these animals from an ethical standpoint? Even if a monkey can experience a remarkable level of sufferance, due to PD experimentation, none has ever shown in non-human primates the same level of sufferance experienced by human PD patients' relatives. Caring for a Parkinsonian relative can significantly increase the possibility to suffer from psychological and psychiatric damage (O'Reilly et al., 1996). Therefore, if the level of sufferance is the parameter that should guide our choice to experiment on non-human primates, there could be no ethical question at all, because the total amount of sufferance experienced by humans would be much greater than the animal's one. More interestingly, as already mentioned, we should consider our choice and behavior on the basis of the moral value we assign to animals. If we accept that animals, and non-human primates in this particular case, have some interest that have some moral weight like, for example, the right to live a free life in the forest, are there degrees of importance in terms of moral status, compared to the interest of humans? If different living beings have different moral status us, as humans, do we consider caring for patients more valuable than caring for animals? We believe that the answer is yes. The researcher feels the appeal to affinity (people above animals), which is understandable as a human predisposition (De Cock Buning, 1995). Even if we assign a moral status to animals, recognising the moral value of their interest, we acknowledge the complex and highly sentimental web of relationships which connect people. This awareness make us decide that it is more valuable to care for patients than to care for animals even if, ethically speaking, this position could raise a series of objections (see Singer, for the concept of "Specism", 1983).

PD is a widespread illness: nowadays about 1% of the population over 55 years of age is affected by this disease around the world (Marras and Tanner, 2004; Wooten et al., 2004). Rodents are, of course, very useful and highly exploited models for the study of PD (for example, see Gerfen et al., 1990; Przedborski and Vila, 2001; but see also Maries et al., 2003; Emborg, 2004), but non-human primates can help us to understand some mechanisms related to this disease, which rodents cannot. For example, as we will better illustrate later in this review, non-human primates are still essential to comprehend the origin, development and possible therapies of motor impairments related to PD, whereas less complex organisms are helpful in better understand some basic molecular and neurological mechanisms characterising this disease. No animal model can replicate the complete picture of complex diseases, such as in the case of PD, but different models can help elucidating some particular aspects of that particular disease. Therefore, in some cases we do not have a comprehensive animal model for a particular human disease. However, this does not mean that the use of animals to find therapies for that disease is fruitless and therefore useless, as some detractors of animal experimentation would put it. It means that the animal model has also to be considered in a more comprehensive methodological picture where the "model", we could say, is represented by the sum of parallel or temporally consequent experimentations, from *in vitro* to *in vivo* to clinical studies.

In summary, PD is an important pathological condition, which affects a large part of the population, for which to understand its causes and to find a possible cure is required. However, important information on different aspects of this disease come from invasive experiments performed on non-human primates, whose use is reason for great ethical concern. Application of the 3R principle to the use of non-human primates in PD research can help to, where possible, improve the quality of life of animal subjects utilised in PD studies, while preserving the quality of the scientific data obtained.

Finally, we remark again that the aim of this review is not to give a detailed illustration of the different models of PD developed with non-human primates. Although a general overview on different non-human primate PD models is offered, we surely do not have the knowledge to engage in such an enterprise. However,

we would like to offer an original point of view on this particular area of research, focused on ethical considerations, that could offer some elements for discussion to improve both the quality of the research and the quality of life for the animals utilised.

2. The 3Rs principle

In 1959 two British academics, William Russell and Rex Burch, published a book destined to become very influential. In their book the two researchers proposed a sort of a recipe to be followed by experimenters who intend to use animals in a particular experimental protocol. This recipe, since then known as the 3R principle, would allow researchers to perform their experiments in a more "humane" way (Russell and Burch, 1959). The 3Rs stands for: Replacement, Reduction and Refinement.

Firstly, the researcher should try as much as possible to replace the animal model with an alternative non-animal model. In the definition by Russell and Burch, Replacement is described as: "any scientific method employing non-sentient material which may, in the history of experimentation, replace methods which use conscious living vertebrates" (Russell and Burch, 1959, p. 69). The notion of Replacement originally offered by the authors was referring to the substitution of an animal experiment by an experiment, method or procedure, which used exclusively non-sentient material. However, Russell and Burch made a distinction between complete Replacement and relative Replacement. With relative Replacement, in some phases of the experimental procedure, the use of animals is still necessary, for instance because animals are killed for organs to derive cells for *in vitro* cultivation. A more contemporary interpretation of relative Replacement includes also the possibility to choose an animal with "less complex" nervous system from the one originally presented: for example, a mouse for a monkey, an invertebrate for a vertebrate. The underlying assumption is that the less sophisticated the neurological development, the less the amount of potential suffering caused by experimental procedures. This concept is debatable for different reasons. On the one hand, there is no general consensus on the fact that a mouse suffers less than a monkey. However, it could be argued that the higher cognitive functions of a monkey add some psychological dimension to the level of sufferance, in relation to a mouse. On the other, there are some neurochemical overlapping between vertebrate and invertebrates, for what concerns pain reception and mediation (Greeenberg and Price, 1983; Nunez et al., 1983).

This notion concerning Replacement is also encoded in the current European legislation on the protection of animals used in experiments (Council of Europe, 1986, Article 7, comma 3): "In a choice between experiments, those which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected".

In the second step, the researcher should try as much as possible to reduce (Reduction) the number of individuals utilised in a certain experimental protocol. This aim can be fulfilled, for example, by using appropriate statistical methods, which can determine the minimum number of animals necessary in a particular experimental design, in order to achieve a statistically significant result using a particular statistical test. Other ways to reduce the minimum number of subjects utilised can be the improvement and advancement of experimental techniques, such as the use of imaging technology, as well as the optimisation of breeding programs.

Finally, Refinement starts when we cannot use complete Replacement techniques, and every device of theory and practice has been employed to reduce to a minimum the number of animals used in a particular experiment. Russell and Burch indicated Refinement as "any decrease in the incidence or severity of inhumane procedures applied to those animals which still are to be used" (Russell and Burch, 1959, p. 64). One of the results of a theoretical study recently carried out by a multidisciplinary group (http:\\www.inemm.cnr.it/animalsee.html), involving biologists as well as philosophers, was the re-definition of this concept as follows: "Any approach which avoids or minimises the actual or potential pain, distress and other adverse effects experienced at any time during the life of the animals involved, and which enhances their wellbeing." (Buchanan-Smith et al., 2005, p. 381). This definition includes all aspects of animal's life in which Refinement techniques can be applied: housing and husbandry, techniques used in scientific procedures, procedural care and experimental design. It also calls for an active role by the researcher in trying to ameliorate the captive conditions of the experimental subjects.

3. The use of non-human primates in Parkinson's disease studies

3.1. Characteristics of Parkinson's disease

Parkinson's disease is a progressive neurodegenerative disorder, characterised by the loss of dopaminergic neurons mainly in the substantia nigra (Forno, 1996; Wichmann and DeLong, 2003). The loss of these neurons causes a deficiency in the production of dopamine, which is a neurotransmitter essential for the control of movements. This deficiency leads, in its early phase, to symptoms such as impaired initiation and poverty of movement (akinesia), and slowness of movement (bradykinesia) (Kish et al., 1988). Later on, with more widespread loss of dopamine interesting different regions of basal ganglia, other symptoms appear, that is among others, cognitive disabilities, sleeping and mood disorders (Adler, 2005; Macht et al., 2005).

3.2. Non-human primate models of PD

Non-human primates are considered a useful model for PD studies (see, for example, Collier et al., 2005) and can reproduce different clinical signs of idiopathic PD, such as rigidity, akinesia and postural instability (Burns et al., 1983).

However, non-human primates as model for the study of PD present some limitations. Bingaman and Bakay (2000) mention four shortcomings: (i) a great inter-individual variability on the response to a particular treatment or therapy; (ii) a spontaneous recovery from a mild or moderate form of the induced disease; (iii) a non-progressive characterisation of the disease induced in the primate model; (iv) the tendency to use young and/or juveniles monkeys, whereas in humans the disease is associated with older age.

Another complication related to the non-human primate model of PD concerns resting tremor, typical sign of PD in humans (Scarmeas et al., 2004). Although MPTP-treated monkeys (see below) show akinesia, rigidity and postural abnormalities, resting tremor is infrequently shown. This characteristic of the non-human primate model is further complicated by species differences. For example, the African green monkey (*Cercopithecus aethiops*) (Taylor et al., 1997) develops a form of resting tremor similar in frequency to the one observed in human. However, the rhesus monkey (*Macaca mulatta*), rarely develops resting tremor, but more frequently action tremor (Nini and Feingold, 1995). This complicates the interpretation of results, and the identification of possible strategies for recovery. Nevertheless, food retrieval tasks are used in non-human primate model to study the combination of tremor, slowness of movements and motor planning disturbances

that affect the fine motor skills of PD patients (Emborg et al., 1998; Kordower et al., 2000). In these tests, therefore, resting tremor appears to be just a component of a more complex picture related to motor difficulties, and it is not the only abnormality studied.

Non-human primates are preferred to rodents in PD studies when, for example, a new therapeutic compound has to be tested before clinical trials. As a matter of fact, we have talked about animal models used to mimic idiopathic PD, but of the same importance are considerations related to the use of animal models to test therapies to be adopted to try to prevent and fight the effects of the disease. Therefore, important requirements have to be fulfilled by a model for the development of efficient neuroprotective strategies: "The model should induce a replicable nigral lesion; the dopaminergic cell-loss should be stable over time without spontaneous recovery; the model should provide a window of opportunity in which the neuroprotective strategy can work" (Emborg, 2004, p. 124).

3.3. The use of MPTP to create non-human primate models of PD

The discovery of the toxin 1-methyl1-4-phenyl1-1,2,3,6-tetrahydropyrine (MPTP), and its effectiveness in producing symptoms very similar to idiopathic PD, has been crucial for the use of non-human primates as models for this disease. MPTP is highly lipophilic and crosses easily the blood–brain barrier. When it arrives in the brain is converted by the enzyme monoamine oxidase-B (MAO-B) in its active form, 1-methyl-4-phenylpyridinium (MPP^+). MPP^+ induces dopaminergic neuronal death and a variety of toxic effects in the cell, including mitochondrial dysfunction, oxidative stress, energetic failure and cell death (Przedborski and Vila, 2001). MPTP creates in a subject both nigrostriatal pathways damages, not obtainable with the use of reserpine in rodents for example, and motor abnormalities similar to idiopathic PD. The latter is not obtainable by using 6-hydroxydopamine (6-OHDA) in rats (Gerlach and Riederer, 1996). However, an administration of 6-OHDA in the intraventricular system induces partial bilateral nigrostriatal lesions (Vu et al., 2000). Rodents result generally less sensitive to MPTP toxicity than monkeys, with mice being also more sensitive than rats (see Emborg, 2004). This has led, generally speaking, to associate the use of MPTP with the use of monkeys.

There exist different MPTP protocols applied to non-human primates. The *systemic lesioned model* consists in the intra-muscular administration of MPTP leading to the bilateral dopamine depletion, and subsequent death, of nigrostriatal cells (Elsworth et al., 1990). A systemic model is the *chronic low-dose administration* of MPTP (Bezard et al., 1997), where the animal receives a daily injection of the toxin, until it reaches a certain score in a clinical rating scale. Another popular MPTP model in non-human primates is the *hemi-lesioned model*. In this case, MPTP is administrated through a unilateral intracarotid infusion (Bankiewicz et al., 1986). The degeneration of the substantia nigra and the depletion of striatal dopamine cells are more extensive than in the systemic model. A *delayed bilateral model* is characterised by the unilateral intracarotid injection of MPTP, creating a hemiparkinsonian condition. Several months later a second treatment with MPTP is provided to produce bilateral Parkinsonism (Smith et al., 1993). Finally, an *overlesioned hemiparkinsonian model* has been described when unilateral intracarotid MPTP is administrated, followed by systemic administration of the toxin (Bankiewicz et al., 1997).

3.4. Species differences

Historically speaking, an Old World monkey is the traditional non-human primate model for biomedical studies, that is, the genus *Macaca*. However, for what concerns PD studies, another cercopithecine monkey appears to be the first Old World monkey model, that is, the African green monkey (Goldstein et al., 1972, 1977). At the beginning of the 1980s MPTP started to be used in rhesus monkeys (Burns et al., 1983; Chiueh et al., 1984; Markey et al., 1984; Zamir et al., 1984). Few years later, other Old World monkeys were added to the list: long-tailed macaque (*Macaca fascicularis*) (Mitchell et al., 1985), Japanese macaque (*Macaca fuscata*) (Crossman et al., 1985), Bonnet macaque (*Macaca radiata*) (Freed et al., 1988), and baboons (*Papio papio*) (Moerlein et al., 1986). Rhesus macaque and baboons have been observed to develop stable Parkinsonian signs, remaining for several years after the administration of MPTP (Hantraye et al., 1993; Smith et al., 1993).

In general, one of the advantages of the Old World species, compared to New World ones, apparently comes from their higher sensitivity to MPTP, where the acute phases appear within minutes from the injection (Petzinger and Jakowec, 2004). For example, in baboons MPTP induces alpha-synuclein aggregation in the substantia nigra, which causes significant and selective damage to dopamine neurons when present in high concentrations (Duda et al., 2000; Kowall et al., 2000).

However, New World monkeys are used as models for the PD as well. Nowadays when speaking of New World non-human primates used in PD studies, the almost ubiquitous species is the common marmoset (*Callithrix jacchus*) (see Eslamboli, 2005, for a comprehensive review on the use of this species in PD studies). This species is commonly utilised as a laboratory model in different areas of biomedical research, including neurobiology, toxicology and immunology (Pryce et al., 1997). As a matter of fact, its use has increased during the past decade, slowly replacing the squirrel monkey as the preferred New World monkey in biomedical research. In the late 1970s the common marmoset appeared as a model for motor impairments (Crossman and Sambrook, 1978; Sambrook et al., 1979), which was an introduction of this species to the following wide use in PD studies. In the 1980s the use of this species became very significant, mainly thanks to the work carried out by Peter Jenner and collaborators (Jenner and Marsden, 1984; Jenner et al., 1986; and see also Jenner, 2003).

There are different reasons for the success of the common marmosets in PD studies, not all of them related with the efficacy of this particular species as mimicking the different aspect of the diseases. For example, *C. jacchus* is small (the weight of an adult ranges between 300 and 500 g), and therefore it requires relatively less housing space; it breeds quite easily in captivity (breeding pairs give birth to twins approximately every 5 months); it is relatively easy to handle (Baker and Ridley, 1987). From a specific procedural point of view Eslamboli (2005) points out that, because of its small size, the common marmoset is a suitable primate model because stereotaxic surgery is as easy as in with the rat: a rat stereotaxic apparatus, with a minor adjustment, can be efficiently utilised.

As with Old World primate species, the administration of MPTP has been and still is the most widely used toxin to cause PD symptoms in a New World monkey. As already mentioned, Jenner has been the first author to describe such procedure in the early 1980s in *C. jacchus*. The injected monkeys were showing clear PD signs, such as Reduction and rigidity in movements and distorted postures, as well as a loss of vocalisation (Jenner et al., 1984). From a histological point of view, these animals were showing a 63–100% loss of striatal dopaminergic terminals.

Other models of PD obtained with the common marmoset include, for example, the administration of the neurotoxin 6-OHDA (commonly used with rats) and the use of viral vectors to over-express the pre-synaptic protein alpha-synuclein (Duda et al.,

2000). The use of this protein to generate PD symptoms is still in the developing stages, but has already given promising results (Kirik et al., 2003).

Therefore, as Maratos and collaborators have stated, the MPTP-treated primates can provide important findings for the effects on nigrostriatal pathway degeneration, but this model must be put into perspective that this is only a model and should not be taken as the complete representation or replication of PD (Maratos et al., 2003).

3.5. The use of non-human primates in neurophysiological studies of PD

Beside the behavioral observations on Parkinsonian monkeys, of pivotal importance are the neurophysiological studies focused on the roles of different parts of the nervous system in the manifestation of the illness.

In this context, in the past two decades, many advances have been made in the understanding of the role of the basal ganglia in PD. Among the first studies, researchers noticed in MPTP-treated monkeys alterations in the neuronal activity *globus pallidus* and sub-thalamic nucleus. An important finding in this case was a significant increase in the firing activity of neurons located in these areas (Miller and DeLong, 1987). It was then suggested that changes in basal ganglia neuronal activity could be involved in the behavioral alterations observed in MPTP-treated animals (DeLong, 1990).

Other studies aimed at understanding the role of the malfunction of particular neurons of the striatum in the PD condition. Striatal neurons, depending on their electro-physiological activity, can be classified as tonically active (TANs). Researchers, through simultaneous recording of neuronal activity in the striatum in PD monkeys, observed that TANs could amplify the *globus pallidus* neuronal oscillations, and therefore could have an important role in the generation of movement disorders observed in MPTP-treated monkeys (Raz et al., 2001).

In the following years models have been developed to illustrate the involvement of different basal ganglia neurons in PD (Brown and Marsden, 1998). These findings are essential both for the understanding of mechanisms implicated in movements disorders, as well as for the development of successful therapies.

In relation to the last point deep brain stimulation (DBS), consisting in a surgical intervention to implant a medical device, called brain pacemaker, has proved to be therapeutically relevant (Kringelbach et al., 2007). Primate and clinical data are essential in order to identify new basal ganglia sites to test the efficacy of DBS (Brown, 2003; Hashimoto et al., 2003; but see also the review by Israel and Bergman, 2008), as well as to identify areas on which to intervene outside the basal ganglia, such as the pedunculopontine nucleus (Jenkinson et al., 2004) and the motor cortex (Drouot et al., 2004).

The majority of these procedures involve delicate and difficult procedures. Usually, the monkey has to be first trained to sit in a restricting chair, and thought to carry on different kinds of task, generally related to motor activity. Then a recording chamber has to be attached to the monkey's skull, in order to reach the areas of the brain, which are of interest for that particular study. Microelectrodes are then advanced in the chosen area, in order to record the neuronal activity. During the completion of the task, the head of the monkey can be immobilised. It is an invasive procedure, not much different from other types of neurophysiological studies but, thanks to these techniques, PD primate research has provided not only theoretical advancements but it has also lead to successful treatments of human patients, as in the case of DBS techniques.

4. The 3Rs principle and PD studies with non-human primates

4.1. Replacement

Possible itineraries for the application of Replacement in the use of non-human primates in PD studies would be the substitution of the use of monkeys by the use of other species instead of primates (relative Replacement), and/or by *in vitro* or other non-animal alternative methodologies (complete Replacement).

4.1.1. Complete Replacement

For what concerns complete Replacement, the use of *in vitro* techniques provide very useful information on basic mechanisms of PD *per se* (see for example, studies on the PD related mechanisms of cell death: Ziv et al., 1994; Leist et al., 1999; Sherer et al., 2002), but do not appear to be able to replace *in vivo* methodology for different aspects of PD studies, and animal models are still necessary to look at behavioral impairments caused by the disease such as damages to fine motor skills, for the monitoring of activity levels, and for clinical ratings (see Emborg, 2004).

In some cases *in vitro* studies are carried out parallel with *in vivo* methodologies, or as a part of a methodological pathway leading to experimentation with animals, and then to clinical testing. For example in a study testing the efficacy of particular agonists at dopamine receptors, the researchers utilised terminal human cells, as well as rats and marmosets. This integrated approach, based on the efficient use of the best model from different *in vivo* and *in vitro* approaches, allowed the researchers to test the therapeutic effects of these drugs on a variety of manifestations of the disease (Millan et al., 2004). Parallel *in vitro* and *in vivo* studies can be carried out on the protection offered by dopamine agonists against MPTP toxic effects (Joyce et al., 2003). Therefore, if we intend complete Replacement as the use of non-animal model *instead* of the use of animal model for the study of PD, it is difficult to find in the related literature examples, which totally substantiate such possibility.

Computer simulations are developed as well. For example, computational model of dopamine delivery have been used to analyse decision-making process in humans (Egelman et al., 1998; Montague et al., 2004), and simulated responses of dopamine neurons have been proposed as reinforcement for spatial learning tasks (Suri and Schultz, 1999). These models look very promising, but their reliability has still to be tested against the use of biological models, utilising non-human primates (as in the case of Suri and Shultz's paper).

Computational models which do not use animal models to be tested do exist. For example, deficits in working memory, observed in PD, can be modelled by computer. For example Monchi and colleagues created a computational model, based on the basal ganglia-thalamocortical system. This system would operate on a simulated behavioral test. Applying "lesions" to the modelled system, the authors were able to investigate deficits in working memory. The simulated test used was the Wisconsin Card Sorting Test (WCST) and the "lesions", that would occur in PD patients, were simulated by subtracting operating "weights" to the different parts of the model, such as the component simulating the activity of the prefrontal cortex (Monchi et al., 2000; see also Amos, 2000). Finally, Taylor and Taylor created a model which, though the simulation of temporal sequence storage and stimulation, represented motor pathways. "Lesions" applied to the model simulated motor deficits observed in PD patients (Taylor and Taylor, 1999).

4.1.2. Relative Replacement

When we deal with possible relative Replacement, we can argue for the use of invertebrates or rodents instead of monkeys,

assuming the level of sufferance being different between these types of animals. We have already mentioned the use of invertebrate transgenic models, such as the fruit fly (*Drosophila melanogaster*). Studies on this insect have been helpful in understanding the importance of particular proteins on the survival of dopamine neurons. For example, different genotypes expressing different levels of alpha-synuclein have been clarifying the role of this protein in the loss of dopamine in the brain, and the suppression of alpha-synuclein toxicity through particular molecular components (Auluck et al., 2002). Therefore, we can affirm that even if invertebrates are very far from the characteristics of PD disease that can be found in mammalian vertebrates, they represent a very important and irreplaceable link between molecular *in vitro* studies and the use of philogenetically higher animal models (Shulman et al., 2003).

In many cases, but depending on the questions asked, invertebrates cannot substitute a vertebrate model for PD studies. Rodent species can be used for a variety of experimental protocols looking at different aspects of PD. However, there are cases in which the use of monkeys over rodents appear preferable, and these relate both to the development of the disease and to the effects of related therapies. For example, young mice present dopamine projections able to recover from the destructive effects of the neurotoxin (Ho and Blum, 1998), whereas in rhesus macaques MPTP induces a profound DA depletion (Tande et al., 2006).

It can be pointed out that, when the same quality of data can be obtained from a rodent and a non-human primate, rodents can be preferred not just in the context of decreasing the level of sufferance imposed by a particular treatment. As a matter of fact, rodents need less space and can be more easily looked after, in terms of energy and time, for what concerns their welfare (that is, the need to provide environmental enrichments). This fact can add to the Refinement value of a particular experimental protocol, in terms of improving housing conditions.

In general, the phylogenetic closeness between humans and monkeys is still considered a strong argument in favour of the use of these animals, as was noted by Forno et al. (1993): "Although rodents have been widely used to study the effects of MPTP, non-human primates appear to express a wider variety of neuropathologic features in response to MPTP and are, of course, much closer to humans from a phylogenetic standpoint" (Forno et al., 1993, p. 600; see also Dirnagl et al., 1999).

Motor impairments created by PD are an area of research, which still significantly benefit from the use of non-human primate models. MPTP-treated monkeys develop akinesia or bradykinesia, rigidity, postural abnormalities; infrequently, as noted before, they show resting tremor, which is characteristic of idiopathic PD. A serious limitation of this model is that the destruction of the dopaminergic cells is acute rather than progressive. This aspect does not allow mimicking in the model the parallel and slow progression of the disease and the gradual insurgence of motor impairments (Jenner, 2003). However, such limitation is unlikely to be soon solved by replacing an MPTP-treated monkey with another animal or non-animal model.

When the aim of a study is to develop new drugs for the prevention and cure of PD effects, generally speaking, initial testing are carried out on rodents. But then, the use of non-human primates becomes necessary to investigate particular aspects related to the effects of these treatments. In particular, the loss of dopamine content in the caudate-putamen regions of the brain is considered the major responsible for the onset of motor impairments. The main treatment is therefore to replace dopamine in the patient, and this is commonly done providing L-DOPA or dopamine agonists: these treatments are very effective in early stages of PD. However, increasing motor impairments and complications do appear as a result of a combination of the progression of the disorder and the long-term effects of the drug treatment (Quinn, 1998; Obeso et al., 2000). Rodent models of PD develop levodopa-induced abnormal involuntary movements, but such motor complications in rodents are less similar to the ones developed in humans than the complications developed in monkeys (Hallett et al., 2005).

It is worth to note that relative Replacement can represent a form of Refinement as well. As a matter of fact, if evidence can suggest that lower species can experience less pain, therefore the general amount of pain causes by a particular experimentation decreases.

In conclusion, it all comes down to the specific question asked in a particular experiment, but the complexity of the illness often calls for the use of a "complex" model. It is our feeling that Replacement strategies are particularly challenging in the case of PD studies. The complexity of the disease does not allow, at the moment, for the Replacement of non-human primates with other vertebrates, or non-animal models, for particular aspects of this illness. As we have mentioned motor impairments and complications, both in relation to the development of the disease and the effects of treatments such as l-DOPA appear to be aspects of PD for which the use of non-human primate is still going to be essential.

4.2. Reduction

Reduction was originally described as: "reduction in the number of animals used to obtain information of a given amount and precision" (Russell and Burch, 1959, p. 64). Therefore, when all of the efforts have been made to replace in some way the original animal model, the researcher should try as much as possible to reduce the number of individuals utilised in a certain experimental protocol. To this aim, the use of appropriate statistical methods can be very useful (Puopolo et al., 1999; Trajstmann, 2000).

The use of non-human primates in PD studies is characterised by small numbers. As a matter of fact, when non-human primates are the models of choice, small numbers are ubiquitous characteristics of the use of these animals in neuroscience studies. Different factors are concurring together to reach such situation. Some institutions can afford the presence of a high number of non-human primates in their animal houses but, generally speaking and compared to rodents, non-human primates are bigger, they occupy a considerable laboratory space, and their maintenance is costly, both in terms of time and money. Furthermore, methodologically speaking, often neurophysiological studies require the subjects to be accustomed to restraining chairs and to be thought to perform a certain number of tasks. This procedure can be demanding in terms of energy and time. Therefore, the researcher tries to get as much as possible information from a limited number of subjects. This is true for PD studies as well.

However, not only practical reasons lead a researcher to use a small of number of individuals. This choice can be influenced by ethical reasons as well, where the researcher feel that working with a limited number of subjects can offer a better possibility to look after the welfare of their subject in an appropriate manner. In PD, due to the debilitating aspects of this disease, this aspect is crucial.

Therefore, both direct and indirect factors are influencing the choice on a particular small sample size in PD studies with non-human primates. This idea is well expressed by Goldstein and colleagues: "This study involved only a small number of animals. In designing the experiment, we had to take into account not only statistical power but also...financial and ethical limitations on treating and maintaining primates with MPTP-induced severe

Parkinsonism" (Goldstein et al., 2003, p. 859). Furthermore, Hallett and colleagues, in a study using 18 rhesus macaques (*M. mulatta*) affirm that: "All efforts were made. . .to use only the number of animals necessary to produce reliable scientific data" (Hallett et al., 2005, p. 504); and again in Collier, where it is stated that efforts were made to use the minimum number subjects necessary to perform valid statistical analysis (Collier et al., 2005).

It is worth to underline that the need to reduce the number of individuals utilised in a particular protocol for ethical reasons, can not undermine the necessity to obtain scientifically significant results: this is a case in which ethics and science must go hand in hand (Vitale and Alleva, 1999).

4.2.1. Reducing the number by reducing variability

A problem in studies of PD using non-human primates lies in the great variability of response to PD inducing treatments, such as MPTP. For example, different studies indicate that in the same protocol different individuals are affected more than others, and that mildly affected individuals can spontaneously recover (Taylor et al., 1997). Heterogeneous experimental groups could call for the increase of the sample size, in an experimental scenario contrary to the concept of Reduction itself. Emborg (2004) suggests a possible methodological solution to this problem. It has been shown that a severe loss of dopaminergic cells is caused by daily injections of 0.3–0.4 mg/kg intra-muscular of MPTP for 5 days, this effective practice nevertheless causes a high degree of variability in the expression of the disease and high mortality (Elsworth et al., 2000). Instead, if the administration of the toxin is scheduled one or two times a week for a longer time, the illness will develop in the experimental group in a more homogeneous fashion, and with a lower rate of mortality (Perez-Otano et al., 1994; Langston et al., 2000). Generally, more homogenous experimental groups require a smaller sample size, and this could favour the application of the concept of Reduction although, in relation to the concept of Refinement problems of animal welfare could arise. Another possibility to reduce response variability to the treatment is to perform intracarotid injection of MPTP, rather than intra-muscular. This method has been successfully used in macaques, obtaining at least 70% rate of success in producing unilateral Parkinsonism, with a near-to-zero mortality. Furthermore, these subjects remained Parkinsonian for years (Emborg-Knott and Domino, 1998). However, again in this case we can see a possible conflict between Reduction and Refinement. On the one hand, the possibility to work on the same subjects for several years decreases the number of individuals to be injected for the completion of the study; on the other, the amount of suffering caused by the long illness induced on these subjects means a prolonged and significant decrease in their level of welfare, somehow against the concept of humane end-point (see, on the interaction between the 3Rs, de Boo et al., 2005).

Another possibility to reduce the number of subjects utilised, and to limit the number of deaths, would be to monitor in a MPTP-treated individual the development of clinical sign. This information could be used to titrate the doses of the toxin, needed to induce a stable Parkinsonian state. This procedure could reduce the number of deaths due to unwanted complications. Needless to say, this procedure would also result in an improvement of the general welfare of the experimental subjects (Refinement of procedures).

4.2.2. Reducing the number of subjects in neurophysiological PD studies

The Reduction of the number of experimental subjects utilised in PD studies, can be reached by utilising techniques that help the researcher to obtain as much as possible information from a single individual.

Simultaneous recording from multiple single neurons can be one of the solutions. Although particularly difficult from a technical point of view, the advantages of such technique can overcome such difficulties. For example, the same number of recordings can be obtained in much shorter time than the single electrode recording, Furthermore, more information can be gained by using multiple recordings, such as the possibility to analyse at the same time the discharge activities of different cells, providing correlation of different neural firing scale (Lee et al., 1998). Therefore, the amount of information that can be obtained from a particular subject is more varied and useful, than using single neuron recording. Finally, the minor time required to obtain the required recording, shorten considerable the frequency and length of experimental session the monkey has to engage in, having therefore an effect in terms of Refinements of procedure.

Baker and colleagues describe two systems for multiple recordings of neural activity. The first one implies the chronical implantation of electrodes, which cannot be moved anymore once implanted. The second system implies the implantation of electrodes transdurally afresh every day (Baker et al., 1999). The choice between the two systems depends on the aim of that particular study. In terms of animal welfare, if the length of the session is a parameter to look at, the former method is faster, because it des not require to look for suitable recording sites at the beginning of a session. However, multiple screwdrivers have been developed, to be attached to a grid over a cranial opening. This method allows chronically implanted electrodes to be moved when needed (Nichols et al., 1998).

The choice of one of the two methods would not appear to have a particular impact on Reduction, because we are already in a context of multiple recording, which is an optimal way to collect more information from a single individual. Furthermore, the use of larger recording chamber could allow the possibility to reach multiples areas of the brain in the same animal.

Another system to conduct multineuron recording in awake non-human primates has been described by Gray and colleagues. Their system includes a recording chamber incorporating a removable internal sleeve. A membrane is stretched across the bottom of the sleeve, providing a seal between the cranial cavity and the external environment. This membrane, besides decreasing the possibility of infections, allows repeated introduction of electrodes into the brain site, without removing the seal (Gray et al., 2007).

Another possibility, used by many researchers, to reduce the number of subject utilised in a neurophysiological studies of PD in monkeys, is to use the experimental subjects by control of themselves. Recordings can be made in the same subjects, that is, the neuronal activity can be recorded in non-treated subjects first and then on the same subjects, made Parkinsonian by the use of the toxin (Rivlin-Etzion et al., 2008).

As in the case for Replacement, Reduction appears to be a delicate issue in PD studies involving non-human primates. The actual use of small number of experimental subjects is dictated not only by ethical, but by practical and economic reasons as well. Furthermore, due to the variability of individual response shown by the same experimental group to the same disease-inducing procedure, the effort to further reduce the number of individuals utilised is difficult to translate into practice. What can be done, instead, is to reason on the procedure utilised to obtain the model, in order to limit the rate of variability and mortality in the experimental subjects, so to make of the available sample the best possible use.

Finally, if we intend the 3R principle in a broader sense, communication and collaboration between different laboratories could help in reducing the total number of subjects utilised. For example, it has been proposed that, in the use of animal models,

just a small part of the animal is utilised (Driver, 2008; see also Still, 1982). For example, in the case of non-human primates utilised in PD studies, once the brain has been analysed, tissues, organs and fluids could be preserved and utilised for other studies in other institutions. Perhaps, good level of planning and communication between institutions studying PD could help in utilising animal subjects to the fullest.

4.3. Refinement

Historically speaking, it is relatively difficult to find in the primatological PD literature of the 1980s some reference to the welfare of the animals involved in the experiments. However, it is worth noticing that significant development has characterised different aspects of the methodologies used in studies, which utilise non-human primates. For example restraining chairs, currently used, aimed at assuring a firm position of the body and head of the monkey during different cognitive tests, has evolved from a rather crude metal and plastic frames (Mason, 1958; Moody et al., 1970; McNamara, 1973), to rather sophisticated and "user-friendly" models, adjusted to accommodate anatomical differences in different species (for a review, see Rennie and Buchanan-Smith, 2006c).

4.3.1. Reference to animal welfare in PD literature

Most authors working on PD, in the methodological section of their published papers in the last decade or so, make reference to local or international guidelines and legislations concerning the protection of welfare in laboratory animals. These norms can be the British Home Office guidelines (http://scienceandresearch.homeoffice.gov.uk/animal-research/publications/publications/guidance/) (Gnanalingham et al., 1995); the Helsinki Declaration (http://www.wma.net/e/policy/b3.htm) (Herrero et al., 1993; Guridi et al., 1996; Barcia et al., 2004); the Animal Scientific Procedure Act (1986) (http://www.archive.official-documents.co.uk/document/hoc/321/321-xa.htm) (Mitchell et al., 1995; Costa et al., 2001; Hill et al., 2004); the National Institute of Health (NIH) Public Health Service Policy on Humane Care and Use of Laboratory Animals (National Institutes of Health, 2002) (http://grants2.nih.gov/grants/olaw/references/phspol.htm) (Forno et al., 1995; Guridi et al., 1996; Bibbiani et al., 2003; Maazloom and Smith, 2006); the European Directive EEC 86/609 (http://europa.eu.int/comm/food/fs/aw/aw_legislation/scientific/86-609-eec_en.pdf) (Escola et al., 2003; Pessiglione et al., 2003; Millan et al., 2004; Hallett et al., 2005); local ethical committees (Baron et al., 2002; Brownell et al., 2003; Anderson et al., 2003; Akazawa et al., 2003; Maazloom and Smith, 2006).

Finally, most of the neuroscience journals specifically require the researchers to indicate to which ethical guidelines or norms their work adhere to. Furthermore, reviewers are often asked to judge on the ethics of the submitted manuscripts.

4.3.2. Refinement in the preparation of different non-human primate models of PD

MPTP, the most common toxin involved in the creation of non-human models of PD is administrated through either intra-venous or intra-muscular injections. Obviously, it is banal to mention, that Refinement of injection procedures would mean to reduce the stress linked with such procedures. The injections should be administrated possibly always by the same people, known to the monkeys, and positive-training techniques should be adopted to minimise the stress related to separation from the group and from the familiar environment (Prescott and Buchanan-Smith, 2007), if the injections need to be performed in a place different from the home-cage.

We could suggest that a Refinement for the procedures would be represented by the Reduction of the number of injections performed. However, it really depends on the type of model the researcher is interested to. For example, the bilateral systemic model (Burns et al., 1983) requires injections of the toxin 4–5 times over 4–5 days. It is considered a good model, since it closely resembles idiopathic PD. The disadvantages come from the severity of the discomfort imposed on the monkey. As a matter of fact, the animals become so debilitated that it makes it difficult to care for them. Furthermore, the subjects become very dependant on the administration of L-DOPA therapy for their survival. A better methodology appears to be the chronic low-dose administration, where the animal receives a daily dose of toxin, until it develops a score of 8 (the scoring system ranges from 0 to 25) following a Parkinsonian monkey clinical rating scale (Bezard et al., 1997). This model as well resembles very much human PD, and it does not show spontaneous recovery as sometimes seen in the bilateral systemic model, but the monkey does not reach the level of debilitation observed in the bilateral model.

The unilateral models, in terms of Refinement of procedures and general welfare considerations, are the potentially best models. The animals remain relatively healthy and can feed by themselves. Furthermore, in terms of Reduction, the controlateral limb can be used as a control (Annett et al., 1992). The main disadvantage of these models is that it less resembles human disease and, therefore cannot always be preferred, simply because asymmetry is not a prominent characteristic of human PD. But, if asymmetry is not conflicting with the question asked and the type of data needed, unilateral models have some advantages over bilateral models, in terms of Refinement of procedures. Unilateral models are also obtained by the use of the toxin 6-hydroxydopamine, injected into the nigrostriatal pathway (Annett et al., 1990, 1995). Usually, the toxin is injected in one hemisphere, while the other is used as control.

4.3.3. Refinement in neurophysiological studies

As mentioned before usually in neurophysiological studies, such as in the case of PD, a monkey has to sit in a restraint chair, while neurons activity are recorded during the performance of a certain task. This practice can potentially be cause of physical, such as skin abrasion and necrosis of ischial callosities, and emotional stress for the monkey (Nakamura et al., 1982). Experienced stress can be shown by physiological effects, inducing elevations in adrenocorticotrophic hormone (ACTH) and cortisol within 15 min from the beginning of an experimental session (Norman and Smith, 1992; see also Morrow-Tesch et al., 1993). Furthermore, urinary corticosteroid has been observed to increase threefold during the first 3 days of experimental session in male rhesus macaques (Mason et al., 1973).

The emotional stress caused by this practice can be mitigated in different ways. The presence of a companion is one possible solution. If the animals are housed in compatible pairs, the presence of the cage-mate can be of some support. This procedure, for example, was carried out at the Wisconsin Regional Primate Research Center, where a chaired headcap-implanted male rhesus macaque was kept as close as possible to his companion (Reinhardt et al., 1989). Physiological data are available which support the positive effect of such practice (Gonzalez et al., 1982; Hennessy, 1984). However, the psychological welfare of the companion individual must be monitored: stressful reactions could be caused by the sight of chaired individual undergoing experimental procedures (see, for example, Langford et al., 2006, for evidence of empathy in laboratory mice).

It is obvious that a certain degree of cooperation must be created between the researchers and the experimental subjects.

This goes in parallel directions: the animal feels more comfortable during the experimental sessions, and the quality of the data obtained from a relatively calm individual is better than from a stressed subject. Needless to say, once the monkeys have been made Parkinsonian, the effort to prevent and diminish the level of discomfort must be doubled. As already said, as much as possible, experiments should be performed by the same personnel, known by the monkeys. Time should be spent with the monkeys outside experimental procedures, in order to gain familiarity with the different individuals. Data show that in marmoset monkeys, daily positive interactions can decrease the frequency of aversive behaviors directed towards the experimenter (Manciocco et al., submitted for publication).

Positive reinforce training (PTR) is nowadays an increased accepted procedure (Prescott and Buchanan-Smith, 2007), which can be applied to all sorts of experimental protocols, including PD studies in monkeys. Monkeys can be trained to move, with no coercion, from their home-cages into a carrying box, to be taken to the experimental room (Prescott and Buchanan-Smith, 2003; Shapiro et al., 2003). Obviously, this can be effective with normal monkeys used as control in PD studies, whereas in Parkinsonian the application of PTR will depend on the state of the illness. Data are available to show the positive effects of PTR. For example, untrained macaques in a blood collection protocols showed a highest levels of white cells in their blood, compared to trained ones, who were not restrained during the experimental procedures (Reinhardt, 1991).

We have already mentioned in Section 4.2.2 the possibility to shorten the duration of recording session, by using multiple recording techniques. We remind that these techniques, although technically difficult, can both reduce the number of needed individuals, by collecting more information in a single subject, and improve the general welfare of the animals, by reducing experimental time. The number of sessions should be the results of a compromise between the experimental needs and the animal's level of welfare, In general, it is preferable to habituate an individual to a rather tight weekly schedule, if possible, rather than dilute the number of sessions across time. Habituation to the same and frequent experimental procedures can alleviate the discomfort created by the procedure itself. If possible, sessions should be carried out in the home-cage, in order to minimise the stress caused by relatively unfamiliar environments.

It must be offered to the monkeys the best possible care in relation to surgical procedures. Post-surgical treatments must be readily available, and personnel must be ready to intervene in the case of unwanted complications (for a review on anaesthesia and analgesia, see Flecknell and Waterman-Pearson, 2001). In our experience, we witnessed marmosets gently grooming cage-mates that were recovering from anaesthesia (Vitale, pers. obs.), therefore the presence of companions can be of some comfort.

Telemetry is nowadays used in different domain of physiological studies. It represents a clear improvement in the Refinement of procedures, because the animal is free to move while data are collected. In non-human primates telemetry systems have been used to record blood pressure, temperature, motor activity, electrocardiograms, and electroencephalograms and so on (see Rennie and Buchanan-Smith, 2006c). In PD studies telemetry systems, especially when precise recording of neural activity is required, are of relatively limited use. However, a telemetry system has been used to study the effect of the stimulation of the pedunculopontine nuclei in MPTP-treated non-human primates. In such study a macroelectrode, complete with a pulse generator (that can be telemetrically controlled), was implanted in a male Rhesus macaque. The same individual was used as his own control. It was possible to turn on the macroelectrode from a distance, without removing the animal from his home-cage, without the need for restraint or sedation. Furthermore, the results showed the possibility to use such a device to treat clinically PD patients (Jenkinson et al., 2004). This particular study looks promising in terms of larger use in telemetry systems in neurophysiological research, including the use of non-human primates in PD studies.

4.3.4. The PD non-human primates during the earlier phase of the disease

All of the guidelines and norms mentioned in the previous section make reference, in a way or another, to the concept of environmental enrichment as a good practice to guarantee an acceptable level for welfare for laboratory non-human primates. In this and following sections we will deal with the concept of environmental enrichment for PD monkeys. Most of the examples provided are specific for PD monkeys, but some other solutions proposed are beneficial both for ill monkeys as well as for fit ones.

As early as in the 1940s, environmental enrichment was used in laboratory animals as an experimental tool in neurobehavioral research. At that time it was also developed for zoo enclosures in response to the abnormal behaviors shown by animals in environments that did not meet their needs. Environmental enrichment was introduced as a concept in laboratory animal care in the '80s, i.e. with a delay of about two decades, compared to the emergence of laboratory animal science as a separate scientific discipline. At that time, the definition of environmental enrichment became more specific and included explicitly the well being of animals as its major goal. We can consider as environmental enrichment any kind of variation introduced in the housing routine and environment of the captive animal, with the aim of improving its level of welfare. Enrichments have been found to reduce the frequency of behaviors indicators of stress and frustration, to improve general health and rate of reproduction, and to improve the performance in cognitive tests (for exhaustive reviews of the issues of enrichments and animal welfare in non-human primates see, for example, Rennie and Buchanan-Smith, 2006a,b,c).

In the PD literature it is difficult to find explicit reference on the use of enrichments as Refinement techniques for Parkinsonian monkeys, but few examples do exist. However, this is absolutely not to say that the absence of reports on Refinement techniques in PD monkeys literature demonstrates automatically the absence of this kind of care and attention in PD studies. Willis and Robertson, in a study looking at the recovery process of MPTP-treated marmosets, state that: "Marmosets were housed individually in wire mesh cages. . .in pairs of adjacent cages and situated in a room such that visual contact by each pair with the two other pairs was possible. As marmosets are vertical movers, the cages were especially designed for this purpose and fitted with logs to facilitate vertical movement and provide environmental enrichment for the duration of the study" (Willis and Robertson, 2004, p. 10). Foster and colleagues, again in the case of the common marmoset, have shown that a small proportion of MPTP-treated subjects can display a "Climbing syndrome" or "Obstinate Progression syndrome" which can last between 2 and 4 weeks, developing after 2 weeks from the last administration of the neurotoxin (Foster, pers. comm.). These subjects did not seem to be deterred by the use of clear Perspex sheets or padding over the metal grids. The results of this behavior were injuries as a result of poorly coordinated jumps, not to mention the likely frustration resulting from continuously trying to climb over the Perspex sheet. Foster and collaborators developed an environmental enrichment aimed at minimising the effects due to this syndrome. The enrichment consisted of an anti-climbing bag (40 cm × 66 cm × 45.5 cm), made out of quilted poly-cotton fabric, in which animals were placed until they stopped showing

climbing tendencies. Furthermore, these researchers suggest the need to recognise the warning signs of the development of such syndrome, such as increased aggression or the tendency to hang form the top of the cage, in order to find effective ways to minimise the risk of serious injuries.

Jackson has provided a series of important suggestion on the welfare of marmosets utilised in PD studies, which special attention on the care for these animals during and post-MPTP treatment. This author lists a series of physical environmental enrichments, which have proved to be successful with Parkinsonian marmosets. Empty marmoset jelly containers and plastic milk containers, with cuts and holes, can be suspended from a perch, so as to provide both a resting area and swinging diversion; these enrichments are appreciated both by normal and Parkinsonian subjects. Other useful objects can be rigid plastic 10 ml syringe cases, with a small hole cut in one side, from which a marmoset can retrieve treats with the use of one hand only (Jackson, 2001). Other classical enrichments for non-human primates, such as swinging perches or plastic tubing can be useful for normal monkeys, but can be hazardous for MPTP-treated animals, especially when they have serious difficulties in moving around. Generally speaking, this fact raises the question how we can judge the appropriateness of certain enrichment. The effect of environmental enrichments on animal welfare can be assessed using a variety of different measures, such as behavioral comparisons under different conditions, or through measures of preference, motivation or emotional state. Physiological parameters of welfare include measures of hormones, heart rate, blood pressure, immune function, body condition, reproduction, and post-mortem parameters (e.g. adrenal weight, ulcers) (Moberg and Mench, 2000; Honess et al., 2005). However, a special attention to PD monkeys should be granted in this case, because motivation to use a particular enrichment could enter in conflict with actual physical ability to make use of that particular enrichment, proved to be successful with fit individuals.

4.3.5. The later phases of the disease

The enrichments just mentioned above are useful for animals, which retain or, thanks to therapeutic treatment, have regained certain degree of motility, but this can be easily not always the case. MPTP-treatment can seriously compromise the feeding and digestive activity of a monkey. Animals may quickly develop a serious disability in feeding by themselves. For example, in a paper published on a relatively rare case of 6-OHDA lesion performed on a non-human primate, the authors documented the need to hand feed the Parkinsonian monkeys by giving several time a day a solution of fortified milk, through the use of a syringe (starting the day after a second surgical lesion). Then, hand feeding was reduced as the animals resumed spontaneously to consume preferred foods (Mitchell et al., 1995). MPTP-treated marmosets need to be hand-fed as well. In one particular study, this occurred on day 2 of the treatment (Jackson, 2001). In this practice usual foods, such as pellets and fruits, must be softened with water, because the disease causes significant difficulties in the motor apparatus involved in gnawing and digesting the food. Additionally liquid diet, administrated and additional calories must be provided. Furthermore, if the particular protocol requires a daily injection of the neurotoxin by the second or third day, some animal may become constipated. Therefore, it is good practice for this kind of protocol to look on a daily basis for faeces in the cage, or to palpate the animals and measure their body weights (see Jackson, 2001 for details on how to keep tracks of the variation in weights and when and how to intervene). During the MPTP treatment animals have to be constantly checked for a decrease in the body temperature and, if it is the case, additional heating must be provided.

Another important point is to try to provide social comfort to individual during the acute phase of the disease. Again Jackson, in the case of the common marmoset, suggests that pairing two individuals showing the same serious symptomatology can have a mutual beneficial effect. As a matter of fact, common marmosets have been shown to recover faster when using dopamine agonists if housed socially (Mitchell et al., 1995). Obviously, in these cases a careful balance must to be reached between the need of the experimenter and welfare considerations.

4.3.6. Conflicts between Refinement and Reduction

Unfortunately, in some cases, the experimenter needs a model in which the seriousness of the disease has to be replicated. A conflict between Refinement techniques, which can mitigate the effects of the disease, and the study of the development and expression of the disease can arise. The challenge is therefore to identify precisely what kind of early condition is needed to answer the specific question asked, to identify predictable early end-points useful for the specific question asked by the study, and to intervene in order to ameliorate the level of welfare of the individuals utilised within the boundaries of the necessary methodology.

Following the administration of the MPTP, monkeys go through acute, sub-acute and chronic phases of behavioral impairments (Jenner et al., 1986; Petzinger and Langston, 1998). Then, with a variability related to age and species, these animals show spontaneous behavioral recovery (Eidelberg, 1986; Kurlan et al., 1991; Petzinger and Langston, 1998). For example in a study by Wichmann and collaborators, three rhesus monkeys were injected with MPTP. Some of the subjects showed striking signs of recovery from the disease, requiring further treatment with the neurotoxin (Wichmann et al., 2001; see also Oiwa et al., 2003). To actively cause a decrement of an improved welfare condition after the treatment with the toxin is clearly against the very concept of Refinement. A solution would be to increase the size of the sample, adding new animals, in order to limit the possibility that too many individuals in the protocol recover from the disease. However, this solution would be against the concept of Reduction, and again a cost-benefit analysis, taking into account both scientific and ethical point of view, would be necessary.

Histological and biochemical analyses are often performed at the end of many investigations on PD with non-human primates, and this is necessary when the degeneration of the neurons interested by the development of the disease has to be examined with accuracy. The link between the behavioral disorders observed and the histological and biochemical damage to the neural systems involved in the disease is fundamental for the development of new therapies (Oiwa et al., 2003; Blanchet et al., 2004; Eslamboli et al., 2005). Therefore, as mentioned before, the question arises whether it is possible to apply humane end-points in order to decide when to sacrifice particular individuals in whom PD has been induced. In the case as well very much depends on the question asked at the beginning of the study. However, very often what is needed is to reach the full manifestation of the disease in order to follow its development, and to observe the efficacy of a particular therapy. Often, it is simply not enough to have just the first signs of the disease, because the question is not whether the disease develops or not, but how it develops with the passing of time. However, having said that, we believe that one important step in the direction of finding humane end-points would be to standardise the scoring methods used in testing the progress of the illness. At the moment, a great variety of scoring methods exist in the literature (see for example Imbert et al., 2000; Chassain et al., 2001). Finding a scoring method, that could identify early signs predictive of the full manifestation of the disease, would be applicable in those studies where the full development of the

disorder is not necessary. The issue of humane end-point is further complicated by different factors, some of them already mentioned: (i) the possibility for some primate species/individuals to recover from the disease, which can add problematic ethical consideration to the very concept of humane end-points; (ii) in the case of MPTP-induced Parkinsonism the choice, mainly based on scientific grounds, whether to adopt a chronic or an acute protocol, which significantly affects the time needed to develop the disease: in some cases experimental individuals are treated for years with the neurotoxin (Herrero et al., 1993; Barcia et al., 2004), and sacrificed years after the first injection (Hantraye et al., 1993; Emborg-Knott and Domino, 1998). All these factors have to be taken into account when reasoning about the notion of humane end-points in PD studies involving non-human primates (for a discussion on humane endpoints, under different aspects, see Hendriksen and Morton, 1999).

Compared with Replacement and Reduction, perhaps not surprisingly (Pollo et al., 2004), Refinement appears to be the "R" easier to implement in PD studies utilising non-human primates. The effort in actively improving the level of welfare of monkeys utilised in PD studies must be directed to all of the phases of the life of an individual: pre-, during and post-treatment. Furthermore, special care and attention is needed when using MPTP to generate the disease: from day to day the symptomatology varies in degrees of seriousness, and different actions to alleviate sufferance must vary accordingly (for a very interesting discussion on the use of behavioral observations to define humane end-points, see Littin et al., 2008).

5. Final remarks and future directions

In this review we have illustrated how the 3R principle can be applied to the use of non-human primates in PD studies. PD is an interesting case study for the application of Russell and Burch's principle because, as mentioned before, in this kind of investigations invasive techniques are used on sentient animals, with potential serious consequences for their state of welfare, but their use is aimed at studying an important and widespread disease seriously detrimental for the health of the human species.

Non-human primates are likely to continue to provide useful models for the study of the origin and development of possible therapies for PD. Researches using animal models should try as much as possible to adhere to the 3R principle (Pereira and Aziz, 2008). Therefore, the important question to be asked is in which ways the 3R principle can be applied to PD studies involving non-human primates.

For what concerns Replacement, the different animal models still hold great credibility in the PD studies community. The complex and different aspects of this illness makes it difficult to switch from animal to non-animal *in toto* (complete Replacement): for example, if cellular studies can help elucidating some mechanisms related to neural degeneration instead of the use of whole organisms, this can still not be the case for the study of motor impairments of this disease. The same goes for relative Replacement: for certain aspects of PD, rodents are still not ready to replace monkeys. In any case, one important aspect to underline is that the choice of an alternative model other than non-human primates for a particular experimental protocol must be justified not only on the basis of ethical considerations, but also with an eye to the suitability of the model to the aims of the research. It must be possible for ethically improved experimental science to remain comfortably within the theoretical and methodological boundaries that define today what is known as good science.

Reduction can be a methodological problem. The number of non-human primates utilised in PD studies is already relatively low, although for some protectionist associations this number could be already excessive. The small number is due to a combination of ethical, economic and practical reasons. Nevertheless, reducing the loss of animals through appropriate protocols could reduce the number of animals utilised (for example using *ad hoc* administration of Parkinson inducing toxins) but, as already noticed before, a tension between Reduction and Refinement could arise and should be discussed.

Furthermore, as outlined above, monkeys present a high level of inter-individual variability in developing the disease, as a response to the same treatment with the neurotoxin MPTP, making it even more difficult a further Reduction of the experimental subjects utilised. However, Reduction of non-human primates utilised in PD studies could be achieved by improving communication between different laboratories.

There is, instead, a considerable possibility to refine the different aspects of the use of non-human primates in PD studies. We have seen, for example, the possibility to help individuals who are particularly impaired by the illness in their feeding activities, and to include in the cage different environmental enrichments as well. This latter procedure has to take into account the motor impairments caused by the disease. A daily observation and care of monkeys affected by PD is essential in order to develop a useful program of enrichment for these particular experimental models.

We would like to summarise some of the points highlighted in this contribution:

(1) The non-human primate models utilised in PD studies still remain a valuable tool to both understand the development of the disease as well as possible new therapies.
(2) The application of the 3R principle has to be tested against different protocols utilised in this area of research.
(3) At the moment Replacement, both complete and relative, suffers of some applicative limitations in relation to PD studies using non-human primates.
(4) Reduction as well, can be problematic in PD studies on non-human primates. However, a better sharing on information between different laboratories, both in terms of positive and negative findings, could ideally reduce the number of individual non-human primates utilised *in toto* in PD studies.
(5) It is possible to refine both the procedure and housing conditions of Parkinsonian monkeys. In the first case, a balance has to be sought between scientific, economic and ethical factors. In the second case, every effort has to be used to improve the state of welfare of PD monkeys by, for example, facilitating feeding activities and introducing *ad hoc* environmental enrichments.
(6) Scoring methods need to be better standardised, in order to identify earlier signs predictive of the full manifestation of the disease, in those studies where the full development of the disorder is not necessary.
(7) Local committees on ethics of research should see the participation of different opinions from different fields of interests. Researchers involved in PD studies should of course be actively involved together with animal welfare specialists, both from veterinary and ethological points of view. Patient advocacy groups should be strongly represented in local ethical committees. Experts in bioethical issues, as well as member of the general public, should be active part of local committees as well.

Finally, with this review we wanted to offer a review of the actual and potential application of the 3R principle in non-human primate models for the study of PD. We believe that although in some case the 3R principle in PD studies shows some applicative

limits, its value, as conceptual and inspirational tool remains extremely valuable. It suggests to the researchers a series of questions, both theoretical and methodological, which can have the only results of improving the quality of life on the experimental models, the quality of the scientific data, and the public perception from the non-scientist community. Therefore, the 3R principle has to be intended as a flexible theoretical and methodological tool, where its applicative value has not been given for granted, nevertheless it remains always a very useful "state of mind" for the animal researcher.

We also believe that the different aspects, which have been illustrated in this review, can represent a useful theoretical background for stimulating cost-benefit analysis on the more general issue of animal experimentation.

Acknowledgements

Fabio Blandini, Hannah Buchanan-Smith, Gemma Calamandrei and, particularly, Giovanni Laviola provided very useful suggestions on earlier versions of the manuscript. We would like to thank Mark Prescott for his advice and useful suggestions. We thank Giovanni Dominici for technical assistance and Jennifer Sienna for checking our English. Finally, four anonymous reviewers provided extremely useful comments and advice. In particular, one reviewer offered us an example of Refinement of procedures, as illustrated in the text. The preparation of the manuscript was supported by a grant to EA (Progetto "Malattie Neurodegenerative", Convenzione n. 533F/A2: "Modelli animali per l'identificazione di markers neurobiologici e comportamentali di insorgenza e progressione del morbo di Parkinson").

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