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Review

The application of Russell and Burch 3R principle in rodent models of neurodegenerative disease: The case of Parkinson's disease

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ABSTRACT

Currently, the accepted ethical standards for the regulation of animal experimentation are provided by the 3R principle (Replacement, Reduction and Refinement). The development of alternative methods to the use of animals (Replacement), the design of adequate experimental protocols to reduce the number of animals (Reduction), the application of refinement practices (Refinement) are all aspects to be considered to ensure ethical and scientific validity to animal experimentation. This review intends to address these issues, using experimental research on Parkinson's disease (PD) as a paradigmatic example of the use of animal models to improve knowledge on a devastating human pathology. In particular, current rodent models of PD and their validity are reviewed and discussed, and methodologies that may ultimately reduce animal's suffering emphasized. Although procedures referring to with 3R principle can be traced in the literature reviewed, they are not considered yet an important part of the methodological information. The formal inclusion in scientific papers of a section devoted to 3Rs may increase knowledge and eventually adherence to this principle by scientists.

Contents

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1. Introduction

Parkinson's disease (PD) is a devastating neuropathological condition, characterized by progressive neurodegeneration of dopamine (DA) neurons in the substantia nigra pars compacta. PD affects a large part of worldwide population – nowadays about 1% of the population over 55 years of age is affected by this disease (Betarbet et al., 2002b; Wooten et al., 2004) – and represents the second most common neurodegenerative disorder after Alzheimer disease (Beal, 2001).

Due to the high prevalence rate of PD, its serious impact on the quality of life of affected individuals and the cost for the community, a great effort is currently made by both experimental and clinical research to clarify the pathophysiology of PD with the aim to develop novel and effective therapies (Kaakkola and Teravainen, 1990; Gerlach and Riederer, 1996; Betarbet et al., 2002a). To this aim, since the beginning of the seventies, a number of animal models, including those involving rodents and non-human primates, have been developed. As PD does not arise spontaneously in animals, its symptomatology is normally induced by administration of various neurotoxic agents destroying specific regions of the brain (Gerlach and Riederer, 1996) or by generating mouse strains with mutations in specific genes (Fleming et al., 2005b). Thus, development of animal models of PD requires extensive manipulation and the use of invasive procedures in sentient animals. The term 'sentient' indicates any species for which it is scientifically recognized the experience of pain and suffering or for which the available scientific evidence is such that this species should be given the benefit of the doubt (Bateson, 1991; Hawkins, 2002). In general, the animal capacity to experience pain or suffering is evaluated on basis of its nervous system, its physiological mechanisms and its behavior (Morton and Griffiths, 1985; Stasiak et al., 2003).

The use of animals to study biological mechanisms of human diseases or to improve medical care is a widespread topic of debate in the scientific community, as well as in the general public, characterized by emotional connotations. Nowadays, the accepted ethical standards for the regulation and review of animal experimentation is provided by Russell and Burch's principle of the 3Rs – Replacement, Reduction and Refinement – (Russell and Burch, 1959, reprinted 1992). This principle has been embodied in guidelines, codes of practice and laws (see for example article 7, paragraphs 2–4 of the Council Directive 86/609/EEC) (CEC, 1986). Application to particular protocols is critical to assess the general value of the 3R principle. Rodent models of PD lend themselves to such analysis, as they represent a paradigmatic example of a balance between costs (in terms of pain and suffering to the animals) and benefit (in terms of new knowledge on the etiology of the disease and potential advantages for human health).

The present review is an attempt to reappraise the methodologies used in basic research within the framework of the 3R principle using rodent models of PD as an example.

2. The disease

Parkinson's disease is characterized by the progressive loss of dopamine (DA) neurons in the substantia nigra pars compacta, an area which plays a critical role in voluntary movement (Dauer and Przedborski, 2003). The loss of DA neurons is paralleled by the accumulation of Lewy bodies, intracytoplasmic proteinaceous inclusions and by a loss of dopamine metabolites, homovanilic acid (HVA) and 3,4-dihydroxyphenylacetate (DOPAC), and of the dopamine transporter in the striatum, as well as in the substantia nigra pars compacta. Furthermore, less extensive neuronal death occurs in other brainstem catecholamine and serotonin nuclei, in hypothalamic neurons, in the nucleus basalis of Meynert and in small cortical neurons (Sherer et al., 2001). Damage to these important neuronal systems may also play a critical role in some of the non-movement-related aspects of PD, such as deficit of cognition and depression (Deumens et al., 2002).

The types of symptoms and their severity depend significantly on the time span since the onset of the disease and the rapidity of functional decline. The cardinal clinical manifestations include bradykinesia, rest tremor, rigidity, gait abnormalities, postural instability. Furthermore, symptoms of depression (Cummings, 1992) and different types of psychosis (Match et al., 2005; Williams-Gray et al., 2006) may be exhibited as well. A good response to treatment with levodopa, a biosynthetic precursor of dopamine, is a clinical feature to distinguish PD from Parkinsonism due to other causes. Compensatory physiological phenomena occur in nigrostriatal system in response to DA neurons depletion, so that a loss of about 80% DA levels in the striatum is thought to be necessary before symptoms become manifest (Hornykiewicz et al., 1986).

The specific etiology of PD is unknown. Vulnerability to neurotoxins or other potential mediators of nigral dopamine cell death is thought to be important in PD's pathogenesis. Postmortem studies have provided consistent evidence for both oxidative damage and a decrease in activity of complex I of the mitochondrial electron transport chain in the substantia nigra (Dunnett and Bjorklund, 1999). Furthermore, in recent years an interaction between environmental and genetic factors in the etiopathogenesis of PD has been suggested (Broussolle and Thobois, 2002).

2.1. Brief review of available rodent animal models of PD: strength and limitations

As mentioned above, the etiology of PD is still largely unknown, and therefore is difficult to identify an ideal animal model to study this disease. Most efforts have been concentrating on the development of animal models with experimentally induced degeneration of nigrostriatal dopaminergic neurons. At the same time, the substantial evidence for involvement of both environmental and genetic factors seems to suggest a heterogeneous etiology, with toxin models more useful in some cases and genetic models in others. Beal (2001) suggested that, for direct relevance to human PD, an ideal animal model should have a normal complement of dopamine neurons at birth with selective and gradual loss of DA neurons initiating in adulthood. The loss should exceed 50% and be readily quantified using biochemistry and neuropathology. The motor deficits, such as bradykinesia, rigidity and resting tremor, should be easily detectable. This model should

show the development of characteristic Lewy bodies and have a relatively short-disease course, allowing rapid and economic screening of therapeutic agents. Finally, in the case of a genetic model it should be based on a single mutation to allow its robust propagation, as well as crossing with enhancer or suppressor strains. However, the general validity of the animal model strongly depends on the research question that the model itself is designed to answer. For example, the gradualness of degeneration might not be essential if the intent of the experiment is assessing the efficacy of an anti-Parkinsonian drug in later stages of the disease.

It is not our aim to provide an exhaustive review of the rodent models of PD, but only to report some paradigmatic examples. The first animal model of PD was derived from the observation that systemic administration of reserpine in rats caused a depletion of dopamine from the striatum, with concomitant hypokinesia, and that this state could be alleviated by levodopa (L-DOPA) (Carlsson et al., 1957). In the following years, several experimental models using domestic animals, like horses (Wang et al., 1991), cats (Schneider and Markham, 1986) and rabbits (Lermontova et al., 1995), have been investigated. Today, the most important species used for PD studies are rodents and non-human primates (Kaakkola and Teravainen, 1990; Jenner, 2003). With regard to the use of rodents, many models have been developed (Gerfen et al., 1990; Emborg, 2004), but those mostly used may be gathered in three main categories: chemical methods inducing degeneration of nigrostriatal dopaminergic neurons (i.e. 6-OHDA, MPTP); pesticide models (i.e. rotenone) and genetic models (Table 1).

2.1.1. Chemical methods inducing degeneration of nigrostriatal dopaminergic neurons

In the preclinical research on PD, intracerebral administration of the neurotoxin 6-hydroxydopamine (6-OHDA) in rat models has been used to induce permanent destruction of the nigrostriatal pathway. The dopaminergic pathway may be lesioned at different levels to mimic PD and the lesions can be unilateral or bilateral. However, the unilateral 6-OHDA model has been, and continues to be, one of most popular experimental models of PD, particularly to investigate the preclinical testing of new therapies and neuroprotective strategies. There are indeed good experimental reasons for performing unilateral dopamine depletion, as each animal can be used as their own control by presenting lateralised sensory stimulation.

As the deficit of dopamine level primarily attributed to the loss of neurons of nigrostriatal dopaminergic pathway is the main biochemical character in PD, this pathway is the brain area targeted by toxin administration. There is a lack of consensus among researchers about the best site for toxin injection. The potential target sites of injections are: medial forebrain bundle (MFB), substantia nigra pars compacta (SNc) and caudate–putamen complex (CPu).

Chang et al. (1999) found that following injection of 6-OHDA into the MFB unilaterally, the DA depletion had to be at least 80% to mimic idiopathic PD. Additionally, both in unilaterally and bilaterally lesioned animals, it was shown that "clinical" symptoms become manifest only when DA depletion in the CPu exceeds 80% (Gerlach and Riederer, 1996). Furthermore, following this kind of lesion not only dopaminergic axons of the nigrostriatal pathway, but also axons of the ventral tegmental area, are damaged (Perese et al., 1989).

Another 6-OHDA model has been developed by using SNc as the target site for toxin injection. In this model, the disease is mimicked more closely with respect to dopaminergic cell loss and relatively small bilateral 6-OHDA lesions of the SNc produce changes in parameters known to be CPu-specific (Van Oosten and Cools, 1999).

As for CPu lesion, this area presents the most profound DA depletions in the brain of PD patients (Nyberg et al., 1983) and since the putamen in humans is equivalent to the ventrolateral section of the rat CPu (Kirik et al., 1998), partial lesions of the ventrolateral CPu are probably the best option. Lesions of this target induce impairments in behavioral parameters, like movement initiation, sensorimotor orientation, and skilled motor behavior (Carli et al., 1985; Sabol et al., 1985; Cousins and Salamone, 1996). In one study with a partial DA depletion (about 80%) in the CPu, paw-reaching deficits and rotational behavior after administration of apomorphine were observed (Lee et al., 1996). This model has been claimed to be a good model of early and moderate stages of PD.

In the past years, several studies have shown that exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a by-product of the synthetic heroin, rapidly causes Parkinsonism in humans (Davis et al., 1979; Langston et al., 1983; Ballard et al., 1985). MPTP converted to MPP^+ (1-methyl-4-phenylpyridinium, the active metabolite of MPTP) accumulates in DA neurons, concentrating

Table 1
Main characteristics of most common rodent models of PD

Animal model	Neuropathological features	Behavioral features	Disadvantages
Chemical models			
6-OHDA	Degeneration of DA neurons in substantia nigra, decreased striatal TH-immunoreactivity (Gerlach and Riederer, 1996; Deumens et al., 2002)	Motor abnormalities, akinesia (Olsson et al., 1995; Barneoud et al., 1995)	Usually unilateral damage (hemi-Parkinson), no Lewy bodies, no gradual loss of DA neurons in adulthood, intracerebral injection (Perese et al., 1989; Deumens et al., 2002)
MPTP	Loss of DA neurons in substantia nigra, decreased striatal TH-immunoreactivity (Beal, 2001; Betarbet et al., 2002b)	Decreased motor activity (Betarbet et al., 2002b)	Not progressive pattern, Lewy bodies are rare, reduced toxin-related susceptibility (Gerlach and Riederer, 1996)
Pesticide model			
Rotenone	Gradual loss of DA neurons in adulthood, reminiscent inclusions of Lewy bodies, decreased striatal TH-immunoreactivity (Betarbet et al., 2000; Sherer et al., 2003)	Flexed posture, reduced activity, rigidity (Betarbet et al., 2000; Sherer et al., 2003; Huang et al., 2006)	Individual-related susceptibility, time-consuming (Betarbet et al., 2000; Sherer et al., 2003)
Genetic model			
Transgenic mice (α-synuclein overexpression)	Loss of DA terminals in the striatum, nuclear and cytoplasmatic inclusions with α-synuclein protein (Fleming et al., 2005a)	Reduced or abnormal motor activity (Rockenstein et al., 2007)	No loss of DA neurons in substantia nigra, expensive and time-consuming (Fleming et al., 2005a)

in mitochondria. In these organelles, it inhibits the complex I of the electron transport chain, causing the production of reactive oxygen species and inducing apoptotic death of DA neurons (Kitamura et al., 2000a; Speciale, 2002). MPTP currently represents the most used Parkinsonian toxin applied in non-human primates (for more details on the issue of non-human primate use in PD, see the companion review paper by Vitale et al., 2008). Rodents, as mice and rats, exhibit in general a reduced sensitivity to MPTP neurotoxicity. As matter of fact, MPTP-induced DA depletion requires a high dose and the permanent behavioral symptoms of Parkinsonism rarely appear (Kopin and Markey, 1988; Kitamura et al., 2000a). However, mice of the C57bl/6 strain are particularly sensitive to this neurotoxin, and can be a useful model, although the most striking difference with PD is the lack of Lewy body formation (Przedborski et al., 2001; Sedelis et al., 2001; Kitamura et al., 2003b).

2.1.2. Pesticide models

Epidemiological studies have suggested that pesticide exposure is involved in the etiopathology of sporadic PD (Gorell et al., 1998; Menegon et al., 1998). In particular, rotenone, a naturally occurring pesticide derived from the roots of particular plant species, is known to be a high-affinity specific inhibitor of mitochondrial complex I (Stryer, 1999; Betarbet et al., 2000; Sherer et al., 2003). It has been found that chronic exposure to rotenone through jugular vein cannulation reproduced many features of PD in rats, including nigrostriatal dopaminergic degeneration and the formation of nigral cytoplasmic inclusions, reminiscent of Lewy bodies. Furthermore, the animals showed bradykinesia, postural instability, unsteady gait and some evidence of tremor (Betarbet et al., 2000). The main limitation of this model is the complexity of the surgical procedure; in fact, the jugular vein cannulation is an extremely labor-intensive surgery. Furthermore, Betarbet et al. (2000) observed both strain and individual variability. In fact, Sprague–Dawley rats showed more variability and less consistent lesion than Lewis rats, and in these latter ones the PD-like pathology was seen only in half of operated rats.

In the following years, Sherer et al. (2003) demonstrated that the same PD symptoms can be reproduced by chronic systemic exposure to rotenone following implantation of subcutaneous osmotic pumps. This protocol, which causes highly selective dopaminergic degeneration and α-synuclein aggregation, represents a substantial improvement in terms of simplicity. In fact, the subcutaneous pump implantation reduced surgery duration from 1.5 h to 0.5 h.

Recent studies have been carried out to assess novel strategies to administrate rotenone. As an example an inexpensive and biocompatible method has been assessed in rats, by direct subcutaneous injections of microspheres releasing rotenone (Huang et al., 2006). This approach produced the typical PD-like behavioral symptoms and immuno-histochemistry assays showed evidence of selective degeneration of the nigrostriatal dopaminergic system, suggesting this method as a feasible one to obtain a reliable PD rodent model.

2.1.3. Genetic models

Although most cases of PD are sporadic, the identification of specific genetic mutations associated with familial forms of the disease and of essential genes for the development of dopamine neurons have led to a new approach in such studies (Jankowsky et al., 2002). Some of the genes implicate in familial forms of PD are: α-synuclein, parkin, DJ-1 and Nurr1. In the last decade, several genetic mouse models (as α-synuclein mice, parkin knockout mice, Nurr1 mice and Pitx3-aphakia mice) have been developed (Hashimoto et al., 2003; Fernagut and Chesselet, 2004; Fleming et al., 2005b). These animals, carrying genetic mutations associated with PD, represent a good tool to investigate the mechanisms of the disease and identify therapeutic targets. The transgenic mice permit to study the interplay between genetic mutations and environmental variables in the etiology of PD. Furthermore, the transgenic models have the advantages to provide insight into the early stages of the disease and to allow investigation of events which lead to the characteristic loss of nigrostriatal DA neurons. Specifically, different transgenic strain of mice carrying α-synuclein mutation display motor deficits, dopaminergic loss and formation of inclusion bodies to a different extent, depending on the gene promoter selected (Rockenstein et al., 2007). However, it is important to emphasize that, although each of these models recapitulates one or various aspects of the familial PD, none of them completely reproduced all the clinical–pathological alterations of this disease.

3. Validity of animal models and the ethical perspective

The use of animals in experimental research requires justification to balance the cost, in terms of pain and suffering to the animals, against the potential benefit, in term of new knowledge and the potential health outcomes for both humans and animals. The cost/benefit principle is a common concept in normative laws on animal experimentation (see for example Animals (Scientific Procedures) Act, edited by Home Office, 1986) and implies that it may be morally acceptable for humans being to use other animals if the benefits deriving for human health justified a certain degree of animal distress or suffering. In this respect, animal models are valuable as models for human diseases to the extent that they adequately represent the diseased state in humans. The rodent models of PD so far described imitate more or less faithfully and extensively the characteristic features of the disease. When a PD-like condition is induced in rats and mice by injection of neurotoxins, the resulting brain damage, as well as motor symptoms, appears shortly after injection. One main limitation of lesion models of PD is the speed of the neuronal degeneration and corresponding functional impairment, that though mimicking the pathogenic mechanisms, does not allow replicating the progressive nature of the human disease. The speed of the process makes it difficult to investigate effectively aspects as the cell vulnerability or the potential benefits of neuroprotective treatments at different stages of the disease. A reasonable strategy for the development of therapies requires early detection of symptoms and initiation of potential neuroprotective treatments as early as possible in order to stop or significantly slow the disease progression. The speed of neuronal degeneration and the associated functional impairment develop at a quite similar pace as in humans in genetic models of PD, when the life-span of rodents is considered. It is thus possible that genetic models, though not reproducing faithfully the etiology of PD, are better suited for assessment of therapies.

The British ethologist Patrick Bateson suggested a set of rules, initially a 'square' (*New Scientist*, 27 March 1980, p. 1002) then a 'cube', to justify or to reject animal experimentation (Bateson, 1986). In the Bateson's 'cube', the quality of research, the certainty of medical benefits and the animal suffering were the issues to be considered. In particular, on basis of this approach, the experimental work on animals should never be done when the degree of animal suffering is high and when the quality of research is low. When the degree of animal suffering is medium, only if research quality is high or if the medical benefits are high, the experiment should be done. When the medical benefits are low, the use of

animals should be allowed only when the quality of research is high and the animal suffering is low.

The aim of the approach of Bateson is to challenge the investigator to ensure that the proposed experiment is of quality and requirement sufficient to justify the total demand that will be made of the animal in term of suffering. In this light, invasive procedures causing pain, suffering or marked behavioral impairment in sentient animals are acceptable as far as the model obtained reproduces more features (i.e. pathogenic mechanisms, neurological phenotype, or behavioral alterations) of the human disease, as to allow for reliable non-clinical evaluation of preventive/therapeutic intervention.

Several other attempts have been made to evaluate the acceptability of animal experimentation. For example, Porter (1992) proposed a model, which was intended as an ethical scoring system to be used by individual scientists as a tool for minimizing animal suffering. The Porter's model is restricted to eight questions, two of which are related to the potential benefits of the study, and six to animal welfare issues. The direct identification of several issues influencing animal welfare and the availability of the required information in the literature might constitute a suitable way to provide a rapid ethical evaluation of any animal experimentation.

3.1. The 3R principle

Nowadays, the accepted ethical standards for the regulation of animal experimentation is represented by Russell and Burch's principle of the 3Rs – Replacement, Reduction and Refinement – (Russell and Burch, 1959, reprinted 1992). In the 1959 (reprinted 1992) Russell and Burch dealt with the subject of the more humane possible treatment of experimental animals. They attempted to establish the general principles of the treatment of the animals during experimentation, taking into account the intimate relationship between humanity and efficiency in animal experimentation. These principles are now enshrined in laws and technical guidelines, and has to be considered in the design and planning of animal experiments.

Replacement means the substitution of animal models with scientific methods employing non-sentient material. Alternative methods, as *in vitro* studies, molecular approaches, mathematical and computer models are included. Russell and Burch also introduced the concept of *relative* Replacement, which describes those methods of Replacement in which animals of lower neurophysiological development or animal tissues are used.

Russell and Burch described the principle of Reduction as reduction in the numbers of animals used to obtain information of given amount and precision (p. 64 Russell and Burch, 1959, reprinted 1992). Efforts for the reduction of the animals used can be pursued by the right choice of strategies in planning and performing the experiments, for example, through accurate experimental design, proper statistical model and analyses, minor duplication of scientific results and better communication among laboratories (Festing and Altman, 2002).

The term Refinement was originally defined as "simply to reduce to an absolute minimum the amount of stress imposed on those animals that are still used". Since then this concept has been extensively redefined. More recently Buchanan-Smith et al. (2005) defined Refinement as "any approach which avoids or minimizes the actual or potential pain, distress and other adverse effects experienced at any time during the life of the involved animals, and which enhances their well-being". This definition suggests to care for the level of welfare of the experimental animal through all of the different stages of its life, and for an active intervention to improve its quality of life.

4. Application of the 3Rs in Parkinson's disease: modelling and research

In the following chapter different protocols employed to study PD in rodent species will be presented and the extent to which they comply with Russell and Burch's concepts of Replacement, Reduction and Refinement discussed (Table 2).

4.1. Replacement

In the study of neurodegenerative disease, such as PD, the exclusive use of alternative approaches appears improbable in the short and medium term. In fact, motor and behavior deficits are major symptoms in Parkinsonian pathology and *in vivo* models are required to investigate the mechanism of disease and its progression. Therefore, in considering the use of alternative methods to enhance our understanding of PD, it has to be taken into account that the reliability and relevance of these methodologies is demonstrated only for specific purposes. For example, *in vitro* models may be a useful tool to understand cell and molecular mechanisms of PD, but they cannot give definite answers on the role played by different structures belonging to the nigrostriatal pathway. Similarly, the mechanisms underlying the motor deficits cannot be studied in invertebrate models, which instead have their greatest value in the field of the genetic approach. However, as in the case of other relevant disease, the integration of findings obtained from different models might be an effective strategy towards a more complete understanding of this complex neurodegenerative disease.

Today *in vitro* model systems and invertebrate models represent the main Replacement approaches in the field of research on Parkinson's disease. In the following sections some of these alternative methods used in PD basic research will be presented and briefly discussed.

4.1.1. In vitro studies

In general, the understanding of the molecular mechanisms causing the disease is the first goal of this kind of models. *In vitro* studies have suggested the involvement of fibroblast growth factor (FGF) in the pathogenesis of PD. For example, Murase and McKay (2006) have found that fibroblast growth factor 20 (FGF-20) promotes survival and stimulates dopamine release in a subset of cells that are preferentially lost in PD. Furthermore, *in vitro* studies have also shown the protective effect of fibroblast growth factor-2 (FGF-2) on DA neurons (Otto and Unsicker, 1990; Shults et al., 2000), suggesting that this role depends on astrocytes (Engele and Bohn, 1991; Krieglstein et al., 1998).

By studies in culture cells, it has been also shown that brain-derived neurotrophic factor (BDNF) plays a role in the survival of DA neurons in the midbrain (Hyman et al., 1991). Furthermore, Hyman et al. (1991) found that BDNF markedly reduces the

Table 2
3R principle and its application in PD studies

Replacement	- *In vitro* studies - Relative Replacement (invertebrate models)
Reduction	- Statistical methodology - Accurate design of experimental plan - Harmonization of experimental procedures - Re-use of animals
Refinement	- Use of genetic models - Consideration of individual variability - Choice of the behavioral test - Skilled experimenter - Improvement of husbandry

cytotoxicity of MPP^+, providing important findings of translational clinical interest.

These kinds of studies are also useful to investigate the role played by environmental pollutants in the development of Parkinsonism. For example, several studies have been carried out to investigate the effect of manganese, a metal widely used in industrial applications. Manganese is an essential element in brain development (Hurley, 1981), but it can be toxic for the dopaminergic system leading to neurological symptoms mimicking Parkinsonism (Archibald and Tyree, 1987; Aschner et al., 2005). In cultured neuronal phenotype PC-12 cells, which are a neuroendocrine cell line with the capability to produce the neurotransmitter dopamine (DA) and contain functional DA metabolism pathways, the exposure to nanoparticles of manganese (Mn-40) produces DA depletion and reduction of its metabolites (DOPAC and HVA) in a dose-dependent manner. Additionally, Mn-40 produces high levels of reactive oxygen species, which suggests that DA depletion may be associated with oxidative stress (Hussain et al., 2006).

Furthermore, a series of *in vitro* studies have been carried out to investigate if elevated levels of iron and monoamine oxidase-B activity in the brain can be major pathogenic factors in the development of PD (Zheng et al., 2005; Gal et al., 2006). These data provide useful information for developing novel neuroprotective drugs for PD related neurodegeneration, which are based on combined iron chelator-monoamine oxidase inhibitors.

In vitro studies investigate also the molecular mechanisms by which mutations in familial-linked genes cause PD. This approach may be promising for unravelling the mechanisms by which DA neurons degenerate in PD. For example, the impairment of the activity of parkin E-3 ligase has been suggested as a possible cause of familial autosomal recessive PD. In fact, this protein might be required for formation of Lewy bodies as these bodies are absent in patients with parkin mutations (Dawson, 2006). Furthermore, a potential link between α-synuclein and both mitochondrial dysfunction and oxidative damage has been suggested by a series of evidences including production of inclusions, morphological abnormalities of mitochondria and increased free-radical production following α-synuclein overexpression in a hypothalamic neuronal cell line (Hsu et al., 2000). Increased markers of oxidative damage are associated with the expression of mutant α-synuclein in human cell line NT2/D1 (Lee et al., 2001). The elucidation of the pathways of oxidative stress and genetic regulation may be followed by development of new therapies for PD and other disorders associated with these events.

The use of *in vitro* studies is accepted to be a valid Replacement strategy, nevertheless it is important not to forget the limitations of this approach. In fact, while the use of neuronal cell cultures may give useful mechanistic information, their predictive validity for human pathologies remains unclear. Advantages and caveats of in vitro methods have been recently reconsidered in the framework of developmental neurotoxicity regulatory testing (Coecke et al., 2007). However, when modelling a complex neurodegenerative disease involving a whole brain pathway, the caveats of in vitro models appear to overweight the advantages. Anatomic and synaptic organization present in the brain cannot be represented in neuronal cultures (either from primary or immortalized lines). Furthermore, the cultures are kept out of their environment, and can be contaminated from external agents (i.e. bacteria); these factors, representing external variables not completely controllable, limit the generalisability of findings so achieved.

4.1.2. Relative Replacement

The ability to harvest tissue that expresses a specific gene allows to conduct *in vitro* studies in parallel with the use of *in vivo* studies, thus permitting a partial replacement. Relative Replacement by use of organic material may be carried out also by establishment and maintenance of rodent tissue banks and rodent-derived cell culture collections. The development of these strategies may optimise the use of the animals. In fact, even if both in tissue banks and cell line collections animals are still necessary, nevertheless a relatively small amount of animals is required. Furthermore, in the cell line collections, the cells are immortalized. The development of these strategies maximize the use of each single animal, as it can be possible to carry out different experiments using organic material coming from the same animal. Therefore, a positive impact on Reduction principle is also addressed.

An additional example of relative Replacement may be attained by using as models animals with lower neurological complexity, for example invertebrates. Recent advances in the understanding of the molecular basis of neurodegenerative diseases and fruit fly *Drosophila melanogaster* genetics represent a valid strategy to improve the knowledge of these pathologies (Sang and Jackson, 2005). Indeed, the engineering of the simple fruit fly has enabled the scientists to create models that have shed light on the pathophysiological basis of PD (Feany and Bender, 2000; Auluck et al., 2002). For example, Feany and Bender (2000) described a fly model of PD using misexpression of wild-type and mutant α-synuclein. These models have molecular and cytological characteristic resembling PD. Furthermore, both wild-type and mutant α-synuclein resulted in progressive motor impairment.

The fly models should be viewed as an efficient genetic system which may permit to isolate genes or screen compounds libraries at a speed that can be difficult if not impossible to accomplish in mice (Sang and Jackson, 2005). In fact, the relatively simple genomic organization of flies (as that of worms) allows experimenters a large number of flies be mutagenized and screened in a short period of time, thus permitting the identification of even rare mutations. For example, homologs of human PD genes, as parkin, have been identified in the *Drosophila* genome and through the generation of mutations the resulting phenotypes have been studied, providing information on the function of correspondent human genes. Conversely, in mammals redundancy phenomena, supplying duplicated versions of the same gene, make difficult the genetic analysis and impractical or impossible the genetic manipulations.

Another invertebrate model potentially useful to investigate the therapeutic strategies is the flatworm planaria (*Dugesia japonica*). This planaria has a high potential for regeneration, and dopamine plays a key role in its behavior. A cloned strain of *D. japonica* treated with MPTP showed autolysis (like apoptosis) and individual death in a concentration- and time-dependent manner. This effect is completely prevented by novel anti-Parkinsonian drugs, such as pramipexole and talipexole (Kitamura et al., 2003b).

As mentioned for *in vitro* studies, the use of invertebrate models presents some limitations. For example, even if fundamental aspects of cell biology are quite similar in humans and invertebrates (i.e. gene regulation, membrane trafficking, synaptogenesis) and mechanisms of neural development are highly conserved, nevertheless the wide evolutionary gap between mammals and invertebrates must be borne in mind. In fact, the lower complexity of the neural networks in invertebrates can determine different physiological responses to neuronal damage. Last but not least, though simple behavioral responses can be analyzed in non-mammalian models, more complex behaviors relying on cortical structures unique to mammals cannot be studied in invertebrates.

4.2. Reduction

Clear justifications for sample size are not reported generally in PD studies. However, whatever one's view on the ethics of experiments on animals, one would agree that the number of animals used should be reduced providing that the scientific validity of data collection and analysis is ensured.

4.2.1. Statistical methodology

An expert use of statistical methodology can be of great help to reduce the number of animals used without sacrificing the scientific aims. The simplest way to reinforce the statistical power is using a greater number of subjects, but statistical power can be also increased by other means. For example, this aim can be achieved by more accurate measurement and better control of the extraneous variables which increase random error.

More attention to statistical methods can reduce the number of individuals used in a particular experiment (for helpful discussion on this issue (see Still, 1982). In fact, the same scientific validity may be attained with fewer subjects if more powerful tests were used (Machin and Campbell, 1987; Cohen, 1988). For example, if the conditions required for the use of a parametric test are met, then it will be better used than the corresponding non-parametric test. A factorial analysis of variance could be used to test the effects of several treatments in one experiment, rather than testing the effects of different treatments in separate experiments. Sequential tests, where the experimenter decides that the recruitment of animals can stop if a predetermined value representing the difference between treated and control groups is reached before N subjects are tested, may help to reduce the number of animals. In fact, if the therapeutic effects are large, fewer subjects will be needed than with conventional designs (Wetherill, 1976). Certainly, the sequential method can be used only when the experimental outcomes appear in a time moderately short. In fact, if the aim of the study is to assess the responses to the treatment over the course of one year, practical considerations may make impossible to design a sequential procedure. Nevertheless, it should be noted that, in the field of PD research based on rodent models, the studies assessing a chronic response to the treatment involve experiments that usually do not last more than few months (Winkler et al., 2002; Al-Jarrah et al., 2007; Schuster et al., 2008).

Furthermore, the reduction of number of animals used may be also achieved by appropriately estimating the number of animals required to detect minimum difference in the variable under study. To this aim, the experimenter should determine the effect size that she/he would wish to be able to detect and should estimate the standard deviations from previous published experiments: it is then possible to calculate, through specific mathematical equations, the number of animals (sample size) needed to test the hypothesis. The type of mathematical equations to be used depends on the experimental design (two or more groups, independent or dependent observations, quantitative or qualitative response variable, etc.) and, therefore, on the statistical test to be used for the analysis of data. For example, to evaluate the effect of a particular kind of physical exercise (i.e. forced, free, endurance, etc.) on the level of DA depletion in bilateral MPTP lesioned C57bl mice, the investigator may plan the experiment as a completely randomized design. She/he may choose to test two independent groups of animals (runners vs. sedentary mice), measuring the outcome variable (striatal DA content) once per animal. In the case that the outcome variable was normally distributed and the variances were homogeneous across groups, the experimenter may choose to analyze data using the two-sided Student's t test for independent groups, with a significance level of 0.05 and a power of 0.90. To calculate the appropriate sample size she/he should then estimate the expected variance within groups from the literature (for example referring to the paper by Tillerson et al., 2003, where the standard deviation of striatal DA content in a homologous colony of bilaterally lesioned MPTP C57bl mice is S.D. = 3.12 pmol/mg, for a mean value = 6 pmol/mg; Tillerson et al., 2003). The experimenter should also specify the minimum difference in mean striatal DA content, between control and treated animals, that would be of biological importance and that, therefore, the experiment should be able to detect. For example, the investigator may wish to detect a difference between control and treated groups of 4 pmol/mg. Therefore, the effect size, expressed in units of standard deviations, would be 4/3.12 = 1.282. At these conditions, the resulting N (sample size) would be 12.79 individuals.

4.2.2. Accurate design of the experimental plan

An accurate design of the experimental plan, in line with the indication coming from clinical studies, may permit to decrease variability and therefore reducing the number of animal subjects. Specifically, in the case of PD investigation, it is important to consider that people in later stage of their lives are affected and that they are mostly males, suggesting a protective role of female hormones For example, it should be noted that the pre-treatment with estrogens, prior to MPTP treatment, reduces the dopamine depletion in the striatal dopamine cells in both female and male mice (Dluzen et al., 1996; Callier et al., 2000), thus indicating an important role of this hormone in the regulation of dopaminergic system. Moreover, gender differences have been also observed in mice treated with MPTP in the striatum, where higher levels of dopamine depletion were found in males when compared to age-matched females (Miller et al., 1998). Therefore, in animal experiments, the male sex could be selected as a first stage of the investigation, and lesions performed in late adulthood, when neuroplasticity mechanisms are less efficient than in the young animals. Additionally, taking into consideration the sex of the animals is particularly important in rodent models, since in these mammalian species several behavioral responses are sexually dimorphic (Branchi and Ricceri, 2004; Calamandrei, 2004).

The age of experimental subjects is particularly important when the MPTP model is used. In fact, the effects of this toxin seem to be strongly affected by age, with a major vulnerability to MPTP in older mice (Kuhn et al., 2003; Ohashi et al., 2006), suggesting that this neurotoxin may be particularly useful for better understanding of role of the aging in the neurodegenerative process.

Tamas et al. (2005) investigated age and sex differences in behavioral and morphological outcomes after 6-OHDA lesions in substantia nigra in rats. They found that both young and aging females are less susceptible to 6-OHDA toxicity than their male counterparts and that aging animals respond to the neurotoxin insult with more severe behavioral deficits. The sex factor has been also investigated in a genetic mouse model of PD, which presents deficits in the expression of specific monoamine transporters. This model is also characterized by moderate motor impairment. This study analyzed if the age-related decline in motor coordination and balance was different specifically as function of sex of the subjects, but no significant effects of sex were evidenced (Colebrooke et al., 2006).

Even if the choice of a given sex, and age at testing may affect the validity of PD rodent models, nevertheless relatively few PD studies have investigated aging animals in preclinical models (Date et al., 1990; Lindner et al., 1999) or when testing neuroprotective strategies (Connor et al., 1996; Collier et al., 1999; Sortwell et al., 2001). More attention is paid to the sex-effect. In fact, by a systematic revision about the sex of animals used as models

through the papers examined for this review, it has been found that the sex of experimental subjects was specified in the 84% of the cases, with exclusively males used in a percentage of 63%, exclusively females in a percentage of 28%, and both sexes in the 9% of the cases. In these latter the sex variable was considered in the statistical analysis. It is worth mentioning, however that examining the sex effects on particular treatment will require using twice as many animals if both males and females are to be tested with adequate statistical power.

Still in the context of Reduction, another source of controlled variability, when multiple-offspring mammalian species such as rodents are used, can be represented by the consideration of the "litter effect". The variability among littermates is usually much lower than that among subjects of different litters. This phenomenon is well known in studies investigating the ontogenesis of behavior or the consequences of developmental drug or toxicant exposure in the offspring (in the field of the behavioral teratology; Chiarotti et al., 1987; Puopolo et al., 1999; O'Dell et al., 2007). This between-litter variability represents a confounding factor, which can mask the differences due to the factor under study on the item of interest (Chiarotti et al., 1987; Puopolo et al., 1999; O'Dell et al., 2007).

A way to control such confounding factor may be to allocate the different treatments to littermates (split-litter design). When the between-litter variability is larger than the within-litter variability (as usually, above all in outbred strains), the split-litter design will allow to point out even small 'treatment effects' and possible inter-litter differences in the treatment response, using a heterogeneous sample of litters.

The performance in behavioral tests used to investigate the PD symptomatology could be affected by the different behavioral traits of animals belonging to different litters. In this case, splitting littermates in the different behavioral tests may allow one the analysis of the relationships between behavioral responses (for example, by means of a Principal Component Analysis), thus identifying possible underlying behavioral factors (such as anxiety, emotionality, sociality). In the same way, animals belonging to the same litter could be used to obtain more valid information about sex-related vulnerability or the individual variability in the response to a given treatment.

Notably to the aim of this review, experimental designs using paired observations (such as those taken on littermates) or repeated measures collected on the same subject, may result in a noteworthy reduction of the required sample size. Indeed, when the correlation between paired observations is greater than or equal to +0.5, the minimum sample size is less than or equal to one-half that required in the case of independent observations. Such reduction can rise up to at least one-forth when using each subject as one-self control.

In the PD papers examined for this review, the litter factor is very rarely considered. Attention to the littermates is presented in some studies using transgenic mice as models (see for example, Fleming et al., 2004, 2006; Callio et al., 2005; Colebrooke et al., 2006), in which the use of individuals belonging to the same litter is specified. However, information provided are not enough to understand if a split approach within the litters has been applied then in treatment administration.

4.2.3. Harmonization of the experimental procedures

The harmonization of the experimental procedures may be a very effective method to reduce the number of experimental subjects. The standardization of scientific designs among different laboratories may permit easier replication of results, increasing validity of findings. In such context, the comparability of procedures is particularly relevant in PD studies. For example, in the 6-OHDA model numerous factors may affect the final outcome. In fact, factors as the location of toxin injection, the number of injections, the concentration and the injection volume, all affect the degree of neurodegeneration produced (Perese et al., 1989). It has been reported that more pronounced deficits were obtained in animals in which the same total dose of 6-OHDA was distributed over three or four sites in the caudate–putamen complex (Kirik et al., 1998). Furthermore, more accurate indications about the site of injection could increase the reliability of the results and thus reduce the number of animals used. Also the simplicity of the surgical procedure may be an aspect which should be considered. The size of brain structures to be lesioned and/or the complexity of the procedure may affect the success of surgery and hence the total number of animals needed to attain robust information. For example, the small size of substantia nigra makes the surgical procedure particularly difficult, increasing the number of animals to be sacrificed, whereas lesion in the caudate–putamen complex can be more easily performed (Deumens et al., 2002).

4.2.4. Re-use of animals

One promising method of Reduction is the re-use of animals in multiple independent experiments. Nevertheless, this approach requires careful scientific and ethical evaluations. In fact, longitudinal experiments are not possible when carry-over effects are expected or when they will be not eliminated from opportune washing-out periods. Furthermore, an accurate experimental design must be planned when the order of experiments is thought to affect the outcomes. The ethical concern involves that re-use of animals should be considered only when the repeated testing will not cause additional suffering and when the previous experiment has not caused severe pain and stress in the individual, as can be in the case of protocols involving adverse and painful stimulation.

From a normative point of view, the actual Council Directive 86/609/EEC deals with this subject, focusing on the prohibition of re-using subjects "in experiment entailing severe pain, distress or equivalent suffering". In a similar way, in UK the Animals (Scientific Procedures) Act (1986) discourages the re-use on the basis of the ethical concern that a primary goal of the regulation must be the protection of the experience of individual animal, and that if the re-use practice causes an additional suffering, then it is considered unethical. In the United States the regulations set forth by the Animal Welfare Act (1990) and the Public Health Service Policy on Humane Care and Use of Laboratory Animals do not provide explicit recommendations for the re-use of animals in preclinical research. In the absence of guidelines or regulations, it is left to individual institutions to determine their own practices for enrolment of an animal in multiple independent experiments. However, it should be noted that protocols involving multiple survival surgeries on single animals require a special scrutiny from Institutional Animal Care and Use Committees (IACUCs). In the case of PD, a potential application of the re-use concept may be obtained by longitudinal analysis on lesioned animals to assess the emergence of the pathology and its progression, including the outcome of treatments. Furthermore, it would also be advisable to obtain brain samples from the same animals for neurobiological investigation. Therefore, rather than using completely independent groups, different kinds of experiments may be carried out using only one single group of animals, with the possibility to obtain a large amount of data from a low number of individuals (see also Still, 1982 for the use of a sequential approach).

The progressive clinical symptomatology presented in the several transgenic mouse models may be particularly useful in this respect. For example, *Engrailed* mutant mice do not show motor impairments in behavioral tests, as open field and grid/hang performance test, at eight months, but they show significant

reduction of performances in these same tests compared with their littermate controls, by 18 months of age (Sgado et al., 2006). Thus, to use the same group of animals, further to reduce the number of subjects used, may allow to verify possible age-related decline in behavioral performances, provided the effects of repeated testing are properly controlled for (i.e. by the use of a small group of unhandled controls).

4.3. Refinement

The definition of Refinement provided by Buchanan-Smith et al. (2005) takes into consideration the avoidance or minimization of *any* suffering, either physical or psychological, certain or potential, at *any* time (before, during and after the experiment) during the life of animal.

4.3.1. The use of genetic models

In the case of PD, the use of genetic models may offer some unique opportunities to enhance animal welfare and to refine the experimental research. Even if "to design" animals to manifest a disease might be ethically questionable (Loew, 1994; Moore and Mepham, 1995; Glenn, 2003), nevertheless there may be a relevant difference if welfare is considered when comparing the potential pain and distress induced by surgery with the production of transgenic mice expressing the disease. In such a framework, the use of transgenic mice expressing the disease may be considered in accordance with the refinement principle.

Furthermore, the ethical implications about the human-made production of transgenic mice expressing a progressively devastating neurodegenerative disease may be discussed. In fact, different levels of ethical concern in producing animals in which the clinical expression of the pathology can range from the full spectrum of symptoms to much milder effects could be raised. In this way, the transgenic rodent may not show serious clinical signs, yet it could represent a good model to the aim of studying disease mechanisms (Brown and Murray, 2006). In this respect, the use of temporal conditional strategies (Polites and Pinkert, 2002; Nagy et al., 2003) can represent another subject of debate. In fact, the use of mice in which the activation/deactivation of the gene-related pathology only emerges at a certain specific time during the animal life-span, can also result in a possible amelioration from a welfare point of view. In fact, this procedure may reduce the length of exposition to stress or discomfort derived from gene manipulation.

In transgenic mice overexpressing wild-type human α-synuclein, the behavioral phenotype worsens with age. In fact, the earlier sensorimotor anomalies appear only by two months of age, when the animals show impairments in motor performance. This profile becomes progressively worse with age and other sensorimotor deficits appear later on by six months. Finally, alteration of fine motor skills are present by eight months of age (Fleming et al., 2004). Therefore, the adoption of careful phenotyping and the accurate definition of endpoints, which can minimize the need for the development of more serious clinical signs in the model, may support the use of genetic mouse models as refinement for a more humane experimental technique.

However, a main limitation to the use of transgenic mouse models for the application of Refinement concept derives from the uncertain outcomes of a genetic program. In fact, beyond the expected clinical manifestations, phenotypic profiles may be affected as a side effect of the genetic manipulation by itself (Mertens and Rulicke, 2000). This consideration should be kept in mind particularly in the case of PD experimentation, where one of the mutations associated with human PD is normally expressed in mice (Trojanowski and Lee, 1999). Through molecular processes, termed pleiotropism and epistasis, undesirable and negatively correlated responses in metabolic, reproductive and in general health processes may be produced. These conditions could involve alterations in physiological or behavioral function, thus limiting the validity of the mouse model.

4.3.2. The issue of individual variability

Besides genetic models, a rodent model of PD with relevant scientific and ethical validity could be obtained by following the strategy proposed by Fleming et al. (2005a,b). In particular, a slow and progressive manifestation of the disease is produced, and in this manner the application of humane endpoints is allowed. In this model, the amount of 6-OHDA neurotoxin that is required to induce a stable deficit is established, step by step, on the basis of the scoring of sensitive behavioral markers. Rats were injected with multiple, escalating doses of 6-OHDA over several weeks. They were then tested for behavioral impairment after each infusion to decide whether another injection was necessary. The goal of the study was to develop an alternative animal model that involved an individual symptomatic threshold to the neurotoxic insult (Fleming et al., 2005a,b). A unique aspect of this study is that, by individualizing the toxin dosage in accordance with the functional outcome of each single subject, a valid experimental model may be obtained avoiding infusion of an unnecessary amount of neurotoxin. This procedure may represent an interesting way to refine animal experimentation in PD research. With regard to the scientific benefits of such a model, the development of a symptomatic threshold, is sensitive to pharmacological applications and may be a valuable tool for use in preclinical research. In fact, the efficiency of potential therapeutic treatments can be assessed at varying stages of degeneration.

4.3.3. The choice of the behavioral test

Behavioral tests represent a valid and very sensitive functional index to determine the extent of neurodegeneration in the animals (Kirik et al., 1998). Several neurological/behavioral tests, including paw reaching, adjusting steps, forelimb-use asymmetry, reaction-time task, Morris water maze task, are used to investigate the extent of the nigrostriatal damage. For example, deficits in paw reaching have been suggested to be similar to the motor deficits seen in patients with PD (Deumens et al., 2002), whereas the adjusting steps test allows the characterization of non-drug-induced deficits in forepaw movement as a model of akinesia and gait problems (Olsson et al., 1995; Lindner et al., 1997). Cognitive impairments are evidenced by learning tasks such as the Morris water maze (Morris, 1981; Whishaw et al., 1985) and the reaction-time task, which provides a good index of motor and cognitive abnormalities even when the dopamine depletion is not severe (Spirduso et al., 1985).

The performance of the animals is strongly affected by the level of nigrostriatal depletion. For example, only a complete DA depletion influences paw-reaching performance, as measured in a skilled motor task in which animals have to reach a food reward using fine, controlled movement of the forepaw (Barneoud et al., 1995; Jeyasingham et al., 2001). Furthermore, depletion of DA levels by over 80% in the caudate putamen, significantly reduces the ability of rats to make adjusting steps, whereas for values of less than 80% no detectable deficits are shown in that particular behavioral paradigm (Chang et al., 1999). These findings point out that, in the framework of PD models, the choice of the behavioral test should be carefully estimated.

For example, Fleming et al. (2005a) looking for an end point of moderate brain damage of 6-OHDA injections chose an appropriate behavioral test when they adopted the limb-use asymmetry test. This paradigm is valid and highly reliable, because its behavioral outcomes are based on a positive correlation with the degree of DA

depletion at a wide range of terminal loss. Thus, the animals that reach a stable score for the behavioral deficits do not need to receive another infusion of 6-OHDA. Therefore, the choice of appropriate behavioral test, which permits the establishment of humane endpoints in the validation of the animal model, enables also to carry out animal experimentation in accordance with the Refinement principle.

The use of behavioral tests sensitive enough to evidence more moderate DA depletions may be particularly advantageous in the development of model for early symptoms of PD (Barneoud et al., 1995; Chang et al., 1999). Moreover, the development of a slow, progressive model of neurological disability together with the choice of suitable behavioral tests could lead to substantial refinement, without reducing scientific validity. This seems to be the case of 6-OHDA bilateral lesions of MFB in the rat. This experimental model has the advantage of mimicking a situation closer to real pathology (PD affects the brain bilaterally) while avoiding the compensatory sprouting of axons from the intact side of the brain (Whishaw et al., 1985; Blanchard et al., 1996). Nevertheless, this model is today rarely used because of the intensive animal care required. In fact, artificial tube to feed the rats is necessary as the animals manifest severe symptomatology, including akinesia, aphagia and adipsia (Ungerstedt, 1971; Deumens et al., 2002). The use of sensitive behavioral tests may allow one to observe neurological deficits after a moderate bilateral toxin infusion, avoiding at the same time extensive lesions and severe clinical signs.

4.3.4. The potential for experimenter expertise to reduce inadvertent suffering

The experience of the experimenter and his/her carefulness towards the experimental subjects has a notable impact in the amelioration or elimination of the factors that induce pain, distress or anxiety in the animals. For example, in research involving surgery pre- and postoperative procedures might be applied to prevent unnecessary suffering or stress.

In the case of PD studies, a number of practices on animal models could be carried out during the surgical procedure to reduce its severity. For example, the administration of medical compounds aimed at preventing physiological events, i.e. mucus secretions that make breathing difficult, could help to avoid pain or discomfort to the animals. Furthermore, the administration of antibiotics, the attention to the housing condition of the operated subject (i.e. the use of care to avoid removal of the cannula), and in general a vigilant post-surgery monitoring, may avoid additional suffering for the experimental subjects.

Nevertheless, a major limit of this application of Refinement concept comes from possible subjective interpretation of the definition of mild, and therefore permissible, suffering (Still, 1982; McConway, 1992). Individual awareness and sensitivity on permissible animal suffering might be better kept under control by including specified criteria in guidelines released by committees of scientific associations and journal boards (see also Wurbel, 2007). This approach could help the researcher in making decisions about the manner to reduce inadvertent pain.

4.3.5. The improvement of husbandry

A good level of animal welfare, and therefore of scientific validity, can be guaranteed not only by the refinement of experimental procedures, but also by accurate care of general husbandry (Poole, 1997). The refinement through the improvement of husbandry and the management systems is currently being pursued and is amenable to ongoing improvement. Whereas Russell and Burch associated the animal suffering concept to experimental procedures mainly (Russell and Burch, 1959, reprinted 1992), today it is generally acknowledged that the housing condition of the animals in the laboratory represents an important potential welfare problem (Wolfle, 2005). Minimum laboratory husbandry standards for rat and mice have been prescribed by the European Community in 1986 (CEC, 1986) and revised in 2007 (CEC, 2007). There is also consensus within the scientific community that enclosures and their enrichment should allow the animals to manifest normal behaviors, since any suffering caused by inappropriate housing will typically be of greater duration than that caused by the experiments themselves (Sherwin, 2002).

It has been observed that stress events are involved in the development of Parkinsonian symptomatology (Snyder et al., 1985). Howells et al. (2005) found that mild stressful events, such as the irregularity of food and water availability or of light phases, can reduce the efficiency of neuroprotective mechanisms. Furthermore, the familiarity with the human handler has been observed to affect significantly the animal performance in the behavioral tests (Chesler et al., 2002; Van Driel and Talling, 2005). Therefore, refinement of housing procedures, as the feeding and cleaning routine, as well as the familiarity of animals with the handler, can be of great relevance for the outcomes of PD studies, by reducing the amount of stress experienced by brain-lesioned rodents.

The social context and the physical furnishing of the environment where the animal subjects live are also important aspects to guarantee a suitable laboratory condition. Besides, these issues are involved in the '*satisfaction*' of the behavioral needs of these animal species (Balcome, 2006). It has long been observed that rats and mice show strong motivation for the company of conspecifics (Jennings et al., 1998; Van den Berg et al., 1999; Van Loo et al., 2001; Patterson-Kane et al., 2002). Individual housing is in fact responsible for physiological and behavioral alterations, inducing stress and behavioral stereotypies (Hurst et al., 1999; Van Loo et al., 2001; Zimmermann et al., 2001; Sharp et al., 2002). Moreover, a general improvement of welfare level has been found in rodents housed in physically enriched cages, where the exhibition of a wider repertoire of natural behaviors has been observed in comparison with standard facility rearing (Townsend, 1997; Jennings et al., 1998; Manser et al., 1998; Van der Harst et al., 1999; Patterson-Kane, 2002; Marashi et al., 2003).

In non-clinical PD research, the evaluation of therapeutic strategies represents another example where the Refinement concept (through the improvement of housing conditions) and the scientific aim can be in accordance. Physical exercise is thought to improve motor function and emotional well-being both in patients with Parkinson's disease (Szekely et al., 1982; Hirsch, 2000) and in experimental animal models (Paylor et al., 1992; Sherwin, 2002; Patterson-Kane, 2002). For the unilateral 6-OHDA rat model and the bilateral MPTP aged C57bl mice, it has been observed that physical exercise, either forced or free, can have neuroprotective effects, as measured by improved neurochemical and behavioral outcomes (Tillerson et al., 2001, 2003; Mabandla et al., 2004). This kind of results is encouraging the introduction of enriched material, including running wheels into the cages, with the aim to improve the welfare levels of animals.

Furthermore, the complexity of the surrounding environment has been demonstrated to affect the rodents' phenotype at different levels (Laviola et al., 2008). In fact, increased environmental complexity has been shown to enhance neurogenesis (Ehninger and Kempermann, 2003) and increase neuronal metabolic activity (Turner et al., 2002). In contrast, impaired brain development is observed in mice caged in impoverished living environments (Renner and Rosenzweig, 1987). These physiological and behavioral changes are thought to affect the learning and

memory performances (Paylor et al., 1992; Woodcock and Richardson, 2000) and the level of anxiety-like behavior (Sherwin and Olsson, 2004), thus modifying the coping response (Patterson-Kane et al., 1999; Zimmermann et al., 2001) and the degree of recovery from brain injury (Passineau et al., 2001). In spite of the advantages for animal welfare represented by enrichment procedures, it has to be considered that these same procedures may bias the results of experiment carried out in animal models of neurodegeneration (Wurbel, 2001; Turner et al., 2002).

In several cases the recovering capacity and the disease progression are significantly affected by housing conditions (Passineau et al., 2001; Hockly et al., 2002; Kobayashi et al., 2002). Therefore, the use of appropriate control groups to guarantee an effective attention to these aspects is particularly required.

Altogether, the risk that environmental enrichment might disrupt the scientific validity of results is a matter of discussion. However, it has been shown that environmental enrichment increases neither individual variability in behavioral tests nor the risk of obtaining conflicting data in replicate studies on mice. This suggests that the housing conditions of laboratory mice can be markedly improved without affecting the scientific validity of data (Wolfer et al., 2004). The reproducibility of results may be guaranteed through standardization of housing among different laboratories. Different degrees of environmental complexity may be established to enable standardized cage furnishing. The observation that merely adding structures to a cage has limited effects on behavior (Olsson and Dahlborn, 2002), could suggest the introduction of some enrichments and not of others. For example, regarding physical enrichments, nesting materials, rather than wheels, or manipulanda objects could be preferred, to not jeopardize the experimental outcome (Van de Weerd et al., 1997).

The evidence that play behavior in rats induce their brains to secrete large amounts of dopamine into the bloodstream (Knutson et al., 1998; Burgdorf and Panksepp, 2001), is another issue to be considered in the design of social enrichment plans for PD animal models. Furthermore, the increase of stressful events, as intraspecific aggressive encounters, in social housing condition (Haemisch et al., 1994; Hurst et al., 1996; Marashi et al., 2003), could be resolved, rather than by isolating the animals, by creative husbandry improvements (i.e. shelters to hide by view of dominant individual, providing more space) or by considering factors, such as the familiarity among the animals, the influence of age, sex, strain and group density. Finally, a specific effect of the strain on the behavioral responses to environmental enrichment has been also evidenced in the mouse. C57bl and BALB/c mice showed increased levels of reactivity and anxiety, respectively, when housed in enriched environments (Van de Weerd et al., 1994). This finding also suggests that a further refinement of procedures should involve the standardization of strains used in comparative studies.

5. The Interplay between 3Rs in the rodent models of Parkinson's disease

Replacement, Reduction and Refinement are often considered separately. However, it should be taken into account that when only one of the 3Rs is independently applied, this choice may have a positive or vice versa negative effect on one or both of the remaining two. Some examples are discussed below with specific reference to studies conducted in PD models.

The care for Refinement principle in PD studies may have positive impact also on Reduction when the standardization of procedures is carried out. For example, variations in cannula placement have been found to influence the number of 6-OHDA infusions required for the production of stable behavioral deficits. In particular, it has been shown that discrete lesions in the dorsomedial striatum do not affect bracing of the controlateral forelimb in 6-OHDA-treated rats, whereas lesions in the dorsolateral region of the striatum have significant effects on bracing (Chang et al., 1999). Therefore, differences in the cannula location may cause different degree of damage and increased variation. That will require a greater number of replications, each of them recruiting additional animals, to confirm a scientific result. Besides, the potential impact of the procedure is influenced by a number of factors related to the animals involved, as genotype, age, nutritional or reproductive status and the environment in which an animal is maintained. Careful standardization and the different steps of the experimental procedure may directly contribute to Reduction, while improving Refinement.

A varied approach including combination of *in vitro*- and *in vivo*-studies to investigate a particular aspect of the disease can have positive impact on both Replacement and Refinement concepts. In fact, the Replacement concept is met by use of no sentient material and the Refinement is achieved by the varied approach, which permits a more complete knowledge of the mechanisms investigated. This approach has been adopted by Sherer et al. (2003), who use three models (two *in vitro*, one *in vivo*) of increasing complexity to demonstrate the involvement of oxidative damage in rotenone toxicity, supporting in this way the evaluation of antioxidant therapies.

An example of negative interaction between two of the 3Rs is when Reduction and Refinement principles are in conflict with each other. This is the case when we consider the individual variability in response to neurotoxin administration. The experimenter may choose to administer a dose that results in lower mortality, but producing motor symptoms in a lower proportion of animals than that observed with a higher dose. This means that a more "humane" attitude requires a higher number of animals to be treated, in order to obtain a statistically significant result. Therefore, a balance needs to be found.

The use of transgenic PD model mice provides an example of Refinement opportunity. A limiting factor, however, could derive from the fact that not all mice produced by this procedure demonstrate key features of the disease under investigation (Betarbet et al., 2002b). In order to obtain the necessary number of subjects with the specific genetic mutation, a huge number of animals must be produced, which is in contrast with the Reduction principle. A possible compromise may be reached conducting a cost–benefit analysis, i.e. by contrasting the number of animals produced and not utilized against the number of those expressing the full set of the pathological phenotype. In addition, as previously mentioned, the potential value of research outcomes for human health should be considered and carefully evaluated, especially in case of innovative studies that requires the use of a large number of animals to obtain a given genotype (for a more general discussion see Bateson, 1986).

6. Conclusion

The use of animals to study biological mechanisms of human diseases or to improve medical care has long been a matter of debate both in general audience and scientific community. Between the opposite positions of being 'no-restriction' absolutists and animal use abolitionists, many scientists probably occupy the middle ground, in the search of a compromise between the need of reliable models of human diseases and the ethical concerns on animal use.

In such context there should be a constant pressure to identify a road map to reduce to minimum experiments which provide suffering and distress in the animals. In fact, also if a steady state of

optimal welfare is unlike to be attained, there must be a requirement for the continual refinement of practices, aimed at minimizing the psychophysiological distress of experimental subjects. The full incorporation of 3R principle in animal experimentation might eventually result in improved methodologies with advantage for general validity of current PD animal models.

In reviewing non-clinical research on PD it is possible to track down some commitments to comply with the 3R principle. For example, vertebrate animal models have been replaced in some cases with the less neurologically developed invertebrates (Feany and Bender, 2000; Kitamura et al., 2003). The consideration of important factors in the experimental design and statistics, such as age (Lindner et al., 1999; Sortwell et al., 2001), sex (Tamas et al., 2005; Colebrooke et al., 2006) and litter effect (Fleming et al., 2004) is expected to reduce variability in accordance with the Reduction principle. More practical concerns on aspects of experimental procedures in rodent PD models (Perese et al., 1989; Deumens et al., 2002), the investigation of individual thresholds for toxin effect in the production of the 6-OHDA rat model, and the efficiency of the behavioral test used (Fleming et al., 2005a,b) have been discussed.

In general, experimental PD models that more closely mimic the disease progression, with attention to the initial stage of this pathology, are increasing. More limited brain lesions and/or lower dosages of neurotoxins, further to offering the possibility of evaluating neuroprotective therapies, reduce the amount of suffering in experimental subjects. Taken as a whole, all these examples indicate that the intimate relationship between a 'humane' animal experimentation and the validity of the scientific results obtained is recognized by most of neuroscientists working in this field.

Nonetheless, it has to be underlined that the reference to national and international animal care guidelines is usually the only sentence in the papers so far reviewed in which concerns on animal welfare are mentioned. In this regard, particularly appropriate appears the recent proposal to include in scientific journals a 3R principle section in Methods section of published papers (Wurbel, 2007). Explanation of the methodologies applied to comply with Refinement and Reduction strategies may represent a useful piece of information for many scientists, as to improve general knowledge on feasible and low-cost techniques to apply the Russell and Burch model when planning and performing experiments.

Furthermore, the adherence to the 3R principle by the biomedical community and the dissemination of the related methodologies may increase the acceptability of animal experimentation by public opinion. A more transparent approach might modify the general opinion that scientists are hostile to issues related to animal welfare. Instead, most of them are interested in promoting and developing refined methods in animal experimentation with the awareness that this attitude might improve the scientific validity of their research. Finally, it is important to note that the feasibility of the 3R principle is pointed out by references to them in laws and regulations. For example, in regard to revision of the EU Directive 86/609, actually in progress, a questionnaire has been sent to experts in the area of animal welfare, animal testing, and animal science to verify, redirect and complete the preliminary findings to be proposed for the revision of the existing legislation. Every subject deals with the questionnaire may be considered a direct or indirect application of one of the 3Rs. This is an example of the great emphasis existing around the need of 3Rs application and diffusion of a "working style" in research where animal welfare and good science go hand in hand.

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