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Unraveling the role of defective genes

Mark R Cookson
Cell Biology and Gene Expression Unit, Laboratory of Neurogenetics, National Institute on Aging, 35 Convent Drive, Bethesda MD 20892-3707, USA. Phone: +1 301 451 3870. Fax: +1 301 451 7295

Mark R Cookson: cookson@mail.nih.gov

Abstract

Several genes that cause familial forms of Parkinson's disease (PD) or similar disorders have been found in recent years. The aim of this review is to cover two broad aspects of the logic of genetics. The first aspect is the recognition that PD can have a genetic basis, either for Mendelian families where genes can be identified because mutations segregate with disease or in populations where more common variants are associated with disease. There are several causal genes for both dominant and recessive forms of parkinsonism, some of which overlap with sporadic PD and some of which have more complex phenotypes. Several of the dominant loci have also been reliably indentified as risk factors for sporadic PD. The second topic is how the study of multiple mutations in any given gene can help understand the role that the protein under investigation plays in PD. Examples will be given of both recessive and dominant genes for parkinsonism, showing how the analysis of multiple gene mutations can be a powerful approach for dissecting out which function(s) are important for the disease process.

Introduction

Our understanding of the underlying cause of Parkinson's disease (PD) has been revolutionized in recent years by the recognition that there are genetic diseases that overlap phenotypically with this common disorder. Although most cases of PD are not inherited, there are many families known worldwide with Mendelian inheritance of diseases that have the characteristic loss of dopamine projection neurons in the substantia nigra that underlies the equally characteristic movement disorder seen clinically in patients. Furthermore, and as will be discussed here, some of the same genes act as risk factors for sporadic disease, suggesting that sporadic and inherited PD share common pathogenic mechanisms.

The focus of this review is on how to take the increasing amounts of genetic data and use it to understand how genetic variants influence protein function. However, it is important to first revisit the genetics of PD and related disorders and to outline briefly how genetic variants can be assigned to be causal.

The genetic basis of Parkinsonism

There are two accepted tests for whether a gene variant can be considered causal for a given phenotype. Either a gene is inherited in a manner that shows segregation with a given trait, usually in a dominant or recessive Mendelian fashion, or a genetic variant shows association with a phenotype in a population. Genes that show segregation tend to be associated with stronger effects on protein function than those that show association, which tend to be subtler.

Mendelian genes for PD show segregation

Of the Mendelian variants in PD, there are several well-characterized genes, two of which show dominant inheritance. In these cases, because we expect to see disease from a single mutated

allele, there is often generation-to-generation transmission of the trait and the disease segregates, or tracks with mutation, for all of the people. A slight issue is penetrance, i.e. what proportion of people with the dominant mutation express the disease. PD is an age-related disorder and the dominant mutations show age-dependent penetrance that, in some cases, seems to be incompletely penetrant even at old age. The first gene discovered for PD was *SNCA*, which codes for the α-synuclein protein, which is a small (14.4 kDa) protein with repeats towards the N-terminus and an acidic 'tail' region at the C-terminus. There are now three point mutations, A53T (Polymeropoulos et al., 1997), A30P (Kruger et al., 1998) and E46K (Zarranz et al., 2004), all in the repeat region. There are also triplications (Singleton et al., 2003) and duplications (Chartier-Harlin et al., 2004; Ibanez et al., 2004) of the entire gene locus reported in different families.

All of these variants, whether point mutations or multiplications, show dominant inheritance and segregate with a Lewy body phenotype that can be similar to either PD or diffuse Lewy body disease (DLBD). Given that α-synuclein is a major component of Lewy bodies (Spillantini et al., 1997), this data supports the general argument that we can define diseases with protein deposition by their pathological outcomes (Hardy, 2005). Penetrance is age-dependent and generally complete for A53T, E46K and the triplications, but appears to be slightly lower in A30P and in duplication families. The latter mutations also appear to give a slightly milder, more brainstem restricted form of PD than the former, which tend to be more like DLBD. Overall, these data show that *SNCA* mutations are a rare but convincing cause of PD/DLBD.

The second dominant cause of PD is the much more recently discovered gene *LRRK2*, which encodes the leucine-rich repeat kinase 2 protein. LRRK2 is a large multidomain protein and there are mutations that segregate with disease in three regions; R1441C and R1441G in the ROC domain (for Ras of complex proteins, a GTP binding region); Y1699C in the COR domain (for C-terminal of ROC), and G2019S and I2020T in the kinase domain (Di Fonzo et al., 2005; Funayama et al., 2005; Gilks et al., 2005; Kachergus et al., 2005; Nichols et al., 2005; Paisan-Ruiz et al., 2004; Zimprich et al., 2004). All of these variants show good evidence for segregation in multiple families and are convincingly causal. There are some non-penetrant cases, particularly reported for G2019S, which is the most common mutation found to date. Specifically, there are case reports including a healthy, older (>90) individual with the G2019S mutation who was free of detectable neurological symptoms upon examination (Kay, Kramer, Higgins, Zabetian, & Payami, 2005). This type of case is important as it tells us why G2019S can be found in apparently sporadic PD; presumably the index patient had one parent with a mutation but the parent never developed PD during their lifetime. Overall, the evidence strongly supports the pathogenicity of LRRK2 mutations, with the important note that there is age-dependent and probably decreased penetrance.

Another interesting observation about LRRK2 mutations is that while clinically the disease is generally similar to sporadic PD (Haugarvoll & Wszolek, 2009), the pathological outcomes can be quite variable, as originally emphasized in one of the first cloning papers (Zimprich et al., 2004). Although most cases examined to date have Lewy bodies containing α-synuclein, some have instead just dopaminergic neuron degeneration and some have protein aggregation that can include the protein tau (Cookson, Hardy, & Lewis, 2008). This is true even within families, where different pathologies are associated with the same mutation. This is perhaps surprising as it implies that the pathological outcome for some has a complex relationship to the gene mutation, unlike the example of α-synuclein discussed above.

One way to resolve this apparent contradiction is to place LRRK2 genetically upstream of deposited proteins such as α-synuclein or tau, implying that the same initial mutation might then result in different pathological outcomes depending on the course the disease takes. There

is some experimental evidence for this (see below), and it is a reasonable interpretation of the available data although it would then be confusing that the same mutation produces similar clinical outcomes. Another thought is that perhaps the final protein deposition (Lewy bodies, tau inclusions etc) is only tangentially related to the clinical phenotype. We might even extend this idea to suggest that while proteins like a-synuclein and tau are involved in the pathological process of LRRK2, their deposition into Lewy bodies or tau inclusions is not required for the disease process. This is an extension of the argument that while Lewy bodies are strongly associated with PD, they may be ancillary to some aspects of the disease process. By extension, the toxic protein species might not be the Lewy body itself but some unidentified version of α-synuclein or tau, perhaps a relatively soluble oligomeric species (Cookson, 2005).

Dominant mutations in *SNCA* and *LRRK2* therefore account for a number of different cases and show the required segregation with disease in multiple families. There is therefore strong genetic evidence that these are causal genes for PD and related pathologies. There are also recessive mutations in three genes, *parkin* (Kitada et al., 1998), *DJ-1* (Bonifati et al., 2003) and *PINK1* (Valente et al., 2004) that show convincing segregation with early onset disease in multiple families. Because these are recessive genes, it is common for each parent to contribute one mutant allele so that there are affected offspring of unaffected parents. In some cases, especially where there are consanguineous marriages (first cousins or similar), the two mutant alleles will be the same, although compound heterozygotes have been reported for all three recessive parkinsonism genes. All subjects who have two mutant alleles are clinically affected and therefore show segregation under a recessive model, although again there is an age-related expression of the phenotype. Mutations in *parkin, DJ-1* and *PINK1* include gene rearrangements (deletion and duplications of whole exons), truncations and point mutations. Deletion and truncation mutations are simple to interpret as loss of function alleles and duplication events often disrupt the protein-coding frame thus effectively removing full-length protein. Point mutations can include those that destabilize the protein for DJ-1 (Miller et al., 2003) and PINK1 (Beilina et al., 2005), thus mimicking loss of function.

One area of controversy is the status of people with heterozygous mutations in *parkin* or *PINK1* who have late onset, typical PD in contrast to early onset recessive disease seen with patients with two mutant alleles. In most of these cases, there is insufficient evidence to say that these mutations segregate in a dominant fashion – parents who would have contributed the mutant allele are not affected with PD and siblings etc are not affected at rates higher than chance alone. Two alternative hypotheses are that single *parkin* mutations might act as risk factors for sporadic PD (Klein, Lohmann-Hedrich, Rogaeva, Schlossmacher, & Lang, 2007) or that the presence of PD in some carriers of recessive mutations might be a coincidence, which could occur relatively frequently in a common disease like PD.

Some of the variants reported might not be pathogenic but rather rare polymorphisms again found at random in patients with a common sporadic disease, e.g., PD. A good example of this are mutations in *Htra2/omi* which were nominated as a gene for PD based on the occurrence in of heterozygous mutations in four cases and not in 500 controls (Strauss et al., 2005). However, subsequent sequencing approaches revealed the nominated mutation (and other variants) in controls (Simon-Sanchez & Singleton, 2008) and a recent study failed to provide support for association of Omi variants with PD (Kruger et al., 2009). Therefore, even though the original data was correct, *Htra2/Omi* is not a gene for PD. One clue that the originally nominated mutations were not causal was that the four cases with PD were apparently sporadic and so there was rather little support for pathogenicity by segregation. In these cases, it is important to sequence a large number of controls to check that the variant is not a rare but benign version of the same gene.

Risk factor genes show association

Some genes do not segregate with disease in families but show association with the given phenotype, i.e., is over- or under-represented in cases versus controls. Because by definition risk variants are present in both disease and controls, assigning pathogenicity is in essence a statistical estimate of the effect. Replication of any apparent initial association in multiple studies is therefore extremely important. A good example of a highly replicated association is ApoE4 variant and Alzheimer's disease, which is consistently near the top of systematic analyses of association studies (see http://www.alzgene.org/). This is because ApoE4 has a strong effect, raising the risk of Alzheimer's disease by about 4 fold, and is a common allele and thus is easy to replicate across studies even with modest numbers of samples (in the 100s).

There are a number of genes that are nominated as showing association with PD and for reasons of brevity we cannot review all of them here (http://www.pdgene.org/ is a useful resource for the interested reader). As an illustrative example, we might consider the data on association of *SNCA* variants with PD. After *SNCA* had been shown to be a gene for dominant Lewy body disease, several groups examined whether common variation around the *SNCA* locus was associated with sporadic PD with both negative (Parsian et al., 1998) and positive results reported (Kruger et al., 1999). With time several additional datasets were collected and collectively supported an association of variants both within the promoter region and towards the 3′ end of *SNCA* with PD (reviewed in Tan, 2007). However, the size of effect of risk variants in *SNCA* is modest, perhaps raising lifetime risk of PD by about 25-30%. Two other genes stood out from these analyses, including variation around the *MAPT*/tau gene and around LRRK2 (Tan, 2007).

One of the limitations of association studies is that one has a preconceived hypothesis; that a given gene is involved in PD, that there is sufficient genetic variation around that gene to be measureable in a given population and that the size of effect is sufficiently strong to be identified in a given number of samples. While this undoubtedly yields insight and can helpfully exclude genes that are not of strong effect, in the last few years methods have been developed to interrogate the genome in a less biased way, using genome wide association studies (GWAS). In GWAS, large numbers of common variants are genotyped in large numbers (typically several 1000s) of controls and cases with the given phenotype. Because the genes are not prespecified, GWAS has the potential to identify novel risk loci for PD.

Two recent studies illustrate the power of this approach, one performed in Caucasian PD patients and controls (Simon-Sanchez et al., 2009) and one in Asian populations (Satake et al., 2009). With a few thousand cases in both studies, each was powered to detect modest associations, in the range of an ~25% alteration in risk for PD which seems reasonable given the data above from prior association studies. Interestingly, in both studies the top ‘hits’ were in and around *SNCA*/α-synuclein. In the study of people from European ancestry, *MAPT*/tau also gave a strong signal (Simon-Sanchez et al., 2009) although this was not seen in the study of people with Asian ancestry(Satake et al., 2009) as the tau gene differs between these two populations (Stefansson et al., 2005). Both studies also nominated a weaker signal around LRRK2, stronger in the Asian population probably because there is a relatively common variant in LRRK2 (G2385R) that is more frequent in Asian populations and that shows robust association with PD (e.g., Farrer et al., 2007).

These GWAS studies therefore nominate genes that we might have expected for PD based on the genetics of Mendelian forms, i.e. *SNCA* and *LRRK2*. But there are a number of surprises. Firstly, new loci were also nominated, including one that has been given the designation PARK16 that contains several candidate genes. Secondly, there was a relatively strong signal for Tau at least in Caucasian populations. Although this had been nominated as a risk gene for PD, because most cases of PD do not have tau deposition it seemed unlikely that *MAPT* would

have as strong of an effect as *SNCA*, but on GWAS the two are close to equal. Thirdly, it was also interesting that the recessive genes were not nominated by GWAS. This does not mean that *parkin*, *DJ-1* or *PINK1* are not genes for PD but rather that the effects of rare variants in these genes are not strong enough at the population level to be measureable in a GWAS design.

Collectively, the evidence from segregating variants has revealed genes of strong effect in rare families and the evidence from association studies show weaker effects in the commoner sporadic form of PD. That these two sets of genetic approaches produce candidates that overlap (*SNCA*, *LRRK2* and perhaps *MAPT*) and in at least one case are also associated with the characteristic protein deposition seen in PD (α-synuclein in Lewy bodies) suggests that familial and sporadic PD may share common pathogenic mechanisms. The next step is then to understand the effects of variation in the nominated genes, using a variety of different models to attempt to put genes in biologically meaningful pathways. As this literature is huge, not all papers on α-synuclein, LRRK2, Tau, parkin, DJ-1 and PINK1 can be reviewed here. Instead, the general principles of how one can take genetic information will be discussed using examples from some of the recent literature on this set of proteins. For clarity, these will be separated into genes for dominant PD/Lewy body disease and recessive parkinsonism. One very important general argument that will be illustrated is that the human genetic data for any given mutation takes priority over supportive arguments for or against pathogenicity from molecular, cell or animal models. As will be discussed, it is critical that independent pieces of weaker data, each of which are ambiguous by themselves, are not allowed to support each other like two drunks standing against each other at the end of the night.

Mutations in recessive genes decrease protein function

Recessive genes usually cause a loss of protein function and we can be reasonably certain that this is the case for *parkin*, *pink1* and *DJ-1* as all three have mutations that segregate with disease under a recessive model that are large deletions. For example, for DJ-1 one of the first reported mutations was a deletion of the entire protein open reading frame (Bonifati et al., 2003). Therefore, we can reasonably assume the understanding the recessive genes requires identifying the normal function of the proteins involved and describing what happens when that function is lost. Therefore, knockout or knockdown models are useful in defining phenotypes related to loss of function genes. An additional approach that can be useful is to use a wide range of different recessive mutations, other than those that are simply unstable or large deletions, and show that they all lack a given property, either a biochemical activity or a phenotype such as protection against toxic stress. In this way, we can be more confident that the identified function or phenotype is relevant for human disease.

An example of using knockouts to define pathways comes from work on the *Drosophila melanogaster* homologues of PINK1 and parkin. In the fly, loss of function alleles of either gene result in a series of age-related phenotypes including male sterility and decreased ability to fly (Clark et al., 2006; Greene et al., 2003; Park et al., 2006). In turn, both of these phenotypes are related to dysfunction in mitochondria. The male sterility seems to be a consequence of failure of spermatids to individualize during spermeogenesis, which is dependent on transformation of mitochondria (Riparbelli & Callaini, 2007), while the flight defects relate to swollen mitochondria in the musculature and apoptosis of muscle cells (Greene et al., 2003).

The mitochondrial phenotypes were perhaps expected for PINK1, which had already been shown to be a mitochondrially directed kinase (Beilina et al., 2005; Valente et al., 2004) with the kinase domain facing the cytoplasm on the outer mitochondrial membrane (Zhou et al., 2008). However, the mitochondrial phenotypes were very intriguing for parkin, which had been suggested previously to be present largely in the cytoplasm, at least under basal conditions (Cookson et al., 2003). Parkin is a protein ubiquitin E3 ligase, responsible for the addition of

ubiquitin to substrate proteins, but none of the reported substrates are known mitochondrial proteins themselves. Furthermore, while mice deficient in parkin or PINK1 do not have dramatic phenotypes, they do have impairment of mitochondrial function (Gautier, Kitada, & Shen, 2008; Palacino et al., 2004). Skin fibroblasts from human cases with parkin (Mortiboys et al., 2008) or PINK1 mutations (Exner et al., 2007) also have mitochondrial impairment.

Therefore, PINK1 and parkin deficiency results in mitochondrial dysfunction across a number of different species but the reasons for this are unclear, especially for parkin. Part of the answer appears to be that parkin can be a mitochondrial protein, but only under specific circumstances. If cells in culture expressing parkin are exposed to carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which allows protons to equalize across mitochondrial membrane and depolarizes the organelle, then parkin can be selectively recruited to the damaged mitochondria (D. Narendra, Tanaka, Suen, & Youle, 2008). Once recruited, parkin then promotes the removal of the depolarized mitochondria by autophagy. Presumably, in the absence of parkin, damaged mitochondria will slowly accumulate in energy rich tissues.

Another surprise was that the phenotype of PINK1 deficient flies could be overcome by increasing expression of parkin, but not the other way around (Clark et al., 2006; Park et al., 2006). Allied to the similar phenotypes caused by loss of PINK1 or parkin function in humans, these results suggest a common pathway with PINK1 genetically upstream of parkin. This work has been extended into mammalian systems by showing that recruitment of parkin to depolarized mitochondria is PINK1-dependent (Geisler et al., 2010; D. P. Narendra et al., 2010; Vives-Bauza et al., 2010), although this does not quite explain how parkin is able to rescue PINK1 deficiency in flies if recruitment to mitochondria is required for function.

Returning to the theme of this chapter, we can now ask how mutations in these two genes influence these functional measures. Using mitochondrial recruitment of parkin as a measure of activity in cells, all recessive versions of PINK1 were shown to be non-functional even those that are stable and expressed at the same level as wild type protein (D. P. Narendra et al., 2010). The only exception is G411S, a variant that has been found in the heterozygous state rather than a homozygous version expected for a recessive allele. It is therefore ambiguous whether G411S is pathogenic. Similarly, recessive versions of parkin either are not recruited to the mitochondrial surface or fail to trigger clearance of mitochondria by autophagy after depolarization (D. P. Narendra et al., 2010).

Taken together, these various studies have identified a series of phenotypes that result from PINK1 or parkin deficiency and show that authentic recessive mutations are non-functional in these assays. For PINK1, it is also reported that the kinase activity is important for function in these assays or in assays of neuroprotection, as artificial kinase dead versions do not substitute for wild type protein (Dagda et al., 2009; Haque et al., 2008; Petit et al., 2005; Sandebring et al., 2009).

However, there are still a series of unanswered questions related to this putative mitochondrial nexus for recessive parkinsonism. Both PINK1 and parkin are enzymes, being a kinase and an E3 ligase respectively so it is critical to understand their substrates, specifically which substrates are responsible for maintaining mitochondrial function and integrity in various systems. There are some reports of a direct phosphorylation of parkin by PINK1 (Kim et al., 2008; Sha, Chin, & Li, 2010) but also negative reports (Vives-Bauza et al., 2010), leaving the most direct possible connection ambiguous. The problem of direct substrates is critical for the development of more direct assays for PINK1 and parkin function.

Another unresolved question is the role of the third gene for recessive parkinsonism, DJ-1. DJ-1 appears to play a role in the control of mitochondrial function, particularly under oxidative circumstances (Blackinton et al., 2009; Canet-Aviles et al., 2004; Dodson & Guo, 2007;

Hayashi et al., 2009; Junn, Jang, Zhao, Jeong, & Mouradian, 2009; Krebiehl et al., 2010; H. M. Li, Niki, Taira, Iguchi-Ariga, & Ariga, 2005; Ved et al., 2005; Zhang et al., 2005). Thus, it seems reasonable that DJ-1 may play similar physiological roles to PINK1/parkin, although DJ-1 cannot substitute for loss of PINK1 like parkin (Exner et al., 2007) suggesting it is either upstream of PINK1/parkin or in a parallel pathway. Finally, it is worth considering why recessive parkinsonism cases have restricted neuronal loss in humans, specifically dopamine neurons of the substantia nigra. All three genes for recessive parkinsonism are widely expressed in most cell types and tissues, so limited expression to one group of neurons can not explain why there is specific cell loss. Furthermore, the mitochondrial phenotype in the flight muscles and spermatids of *Drosophila* says that phenotypes of PINK1 or parkin deficiency are probably not due to dopamine metabolism or neuronal activity *per se*, with the caveat that this is a different species so there may be fundamental aspects of the biology that are not conserved. One possible candidate for sensitivity to loss of recessive parkinsonism genes is ATP utilization by mitochondria under aerobic conditions. There is evidence that flight muscles in *Drosophila* are particularly sensitive to superoxide radicals generated by mitochondria (Godenschwege et al., 2009). The sensitivity of dopamine neurons to toxins such as rotenone and MPTP that inhibit ATP production and result in ROS production may also hint at that there may be similar reasons for apparently disparate phenotypes across species, although this remains speculative and difficult to test if mouse models lack robust phenotypes.

These various data show that understanding the recessive nature of inheritance in early onset parkinsonism helps us set up models that are instructive to understanding normal function and, from there, to show how mutations might lead to disease.

Mutations in *SNCA* and *LRRK2* alter protein function

If this logic is appealingly simple for recessive mutations, the situation for dominant genes is much more complex because here we cannot be sure if normal function of the proteins is at all relevant to the disease process. This is because dominant mutations can have mechanisms such as gain of novel function that are unrelated to the normal role of the protein, as shown for superoxide dismutase mutations relevant for familial amyotrophic lateral sclerosis (Bruijn, Miller, & Cleveland, 2004). However, clues to pathogenic mechanisms can be obtained by again considering what makes mutations similar to each other.

Perhaps the best example of this comes from studies of α-synuclein protein chemistry *in vitro*. Like other proteins that are deposited in neurodegenerative diseases, α-synuclein can acquire a beta-sheet like structure in some conditions and aggregates into higher order aggregated species (Cookson, 2005). Interestingly in the context of mutations that increase protein expression without changing amino acid sequence, such as the duplication and triplication alleles, protein aggregation is a concentration-dependent phenomenon (Giasson, Uryu, Trojanowski, & Lee, 1999; Wood et al., 1999) and therefore simply having too much protein may trigger aggregation and mimic the effects of point mutations.

Both the A53T (Narhi et al., 1999) and E46K mutations (Greenbaum et al., 2005) increase the potential for α-synuclein to aggregate in these *in vitro* models. Interestingly, A30P can actually slow the formation of mature fibrils, the end product of aggregation reactions that may represent the deposited species in Lewy bodies. The shared property of A30P and A53T is the increased formation of oligomers, which are relatively soluble, partially aggregated species formed on the pathway to fibril formation (Conway et al., 2000).

Therefore, if we follow the logic that shared properties of mutations are more likely to represent authentic pathogenic mechanisms then oligomer formation is a good candidate for a toxic event to mediate the toxic effects of α-synuclein. There is some support for this concept from cell culture models where soluble oligomers can be identified (Xu et al., 2002) and where oligomers

can be toxic when applied to the outside of cell membranes (Danzer et al., 2007), possibly through a pore-like mechanism (Kostka et al., 2008).

Although this has not been verified using *in vivo* models, it therefore seems reasonable from the shared behavior of mutant proteins *in vitro* that oligomer formation is toxic to neurons. However, this logic is a little uncertain in part because it relies on the behavior of the A30P mutation that is found only in one small family, and at apparently decreased penetrance. Therefore, interpretation of these mutations requires some caution. This is particularly complex when there are also clear dosage effects and the wild type protein can be toxic in humans.

Another example of the complexity of understanding dominant mutations is LRRK2. LRRK2 is a complex protein but as it contains two possible enzymatic activities, a kinase domain and a GTP binding region, that contain dominant pathogenic mutations, it seems reasonable to examine which of these contributes to pathogenicity. Several studies, admittedly using simple *in vitro* systems, suggest that all mutations in LRRK2 are toxic when expressed at high levels in cultured cells (Greggio et al., 2006; Greggio et al., 2007; Iaccarino et al., 2007; Jorgensen et al., 2009; MacLeod et al., 2006; Smith et al., 2006; Smith et al., 2005). At this first approximation, this toxicity appears to be similar irrespective of whether mutations are in the GTP binding region (e.g. R1441C/G), the kinase domain (G2019S and I2020T) or in the intervening COR sequence (Y1699C). It is therefore interesting to ask whether these mutations really share similar mechanisms at a biochemical and cellular level.

One obvious experiment is to measure how different pathogenic mutations affect kinase activity. Although there is some variation from study to study, the overall picture is that while G2019S in the kinase domain increases kinase activity by about 2fold, the remaining mutations have no significant effect (Greggio & Cookson, 2009). Therefore, altered kinase activity is not a consistent effect of mutations in this domain. But the acute toxicity of mutant LRRK2 is dependent on kinase activity (Greggio et al., 2006; Smith et al., 2006). How can we reconcile the similar effects of different mutations if they are in distinct domains of the same protein and if they have differential effects on kinase activity?

One idea is that the assays that most groups have used are not measuring the correct substrate. Several labs initially measured kinase activity with autophosphorylation, which many kinases will perform *in vitro* but may be a consequence of high concentrations of enzymes in the test tube. Therefore, autophosphorylation may not be a true physiological activity and results may be biased by using the wrong readout. Although several alternate substrates to autophosphorylation have been proposed, to this point none are proven to be physiological either (reviewed in Taymans & Cookson, 2010). And in any case, when kinase activity of LRRK2 is measured with heterologous substrates, then the results are largely similar as for autophosphorylation (Greggio & Cookson, 2009) suggesting that the effects are general to mutations and not dependent on the precise assay conditions.

Mutations in the ROC region, which has measureable but weak GTPase activity (Lewis et al., 2007; X. Li et al., 2007), tend to have lower GTPase activity. It has been suggested that GTP binding to LRRK2 or its homologue LRRK1 can stimulate kinase activity (Korr et al., 2006; Smith et al., 2006). Therefore, one might predict that there are circumstances where LRRK2 might have increased kinase activity for mutations outside of the kinase domain, if the GTP bound state of LRRK2 is the more active and more toxic version and if mutations outside of the kinase domain slow turnover from GTP to GDP. However, there is little evidence yet that this happens and the basic data that GTP stimulates kinase activity of LRRK2 has been challenged recently (Liu, Dobson, Glicksman, Yue, & Stein, 2010).

An alternative view is that the kinase activity of LRRK2 might regulate GTP binding and/or GTPase activity. Support for this idea comes from three recent studies identifying that LRRK2

can phosphorylate its own ROC/GTPase domain (Gloeckner et al., 2010; Greggio et al., 2009; Kamikawaji, Ito, & Iwatsubo, 2009), leading to the proposal that kinase regulates GTPase activity. This is reasonable if LRRK2 is a dimeric kinase that autophosphorylates within the dimer, as suggested elsewhere (Greggio et al., 2008; Sen, Webber, & West, 2009), but only if that activity is physiologically relevant, which is not yet proven.

The overall message about LRRK2 is that while it is feasible to measure at least surrogates of the two major enzyme activities for this protein there are still difficulties in resolving both of these into a simple model for pathogenesis with a shared single output for all mutations. Clearly, a major challenge for the field is to identify the authentic outputs of LRRK2 kinase or other activities, and to try and model the pathogenesis of the human condition.

One area where some recent progress has been made is in understanding the relationship between LRRK2 and other dominant forms of PD. Mouse models have been developed that express mutant forms of LRRK2 in the brain, including a BAC driven R1441G line (Y. Li et al., 2009) and a transgenic G2019S cDNA mouse (Lin et al., 2009). The first animal model is especially interesting because although phenotypes in the lines were generally mild, there was evidence of accumulation of tau in axons (Y. Li et al., 2009). The second animal model showed that there is an additive effect of expressing mutant forms of LRRK2 and α-synuclein (Lin et al., 2009). Furthermore, knockout of LRRK2 limits the toxic effects of mutant α-synuclein suggesting that the effects are specific and not simply due to overexpression of two toxic proteins in the same cells.

These results are important because they show that there are causal relationships between the two genes implicated in the genetics of PD, α-synuclein and LRRK2, and further suggest a role for tau in the same pathogenic pathway. Although the models are imperfect – none have frank degeneration of dopaminergic neurons in the substantia nigra – they reinforce the concept that not only should we examine multiple mutations in the same gene, we should also examine the interactions between genes that produce similar phenotypes in patients. By extension, this leads to the much more difficult question of asking how genes that show association with PD affect lifetime risk of disease.

Risk variants found in association studies likely have subtle mechanisms

As discussed above, recent GWAS studies have reinforced two previously nominated genes that appear to increase lifetime risk of PD, *SNCA*/α-synuclein and *MAPT*/tau, with several other genes of similar effect size being present in the human genome notably LRRK2 and the PARK16 locus. A significant challenge is to understand why these different genes influence disease risk, particularly when with association studies it is not always clear if the nominated variant (usually a single nucleotide polymorphism or SNP) is actually causal for disease. SNPs are inherited in relatively large linkage disequilibrium blocks (as is the case for PARK16) and knowing which gene is the causal variant is therefore difficult. Furthermore, not all SNPs change protein sequence, so for many it is difficult to determine which is most likely to have a biological effect. Occasionally, there are hints as to ways in which genes might affect risk. For example, the nominated *MAPT* risk variants appear to increase tau mRNA expression (Simon-Sanchez et al., 2009). This suggests that having more tau without it being deposited may be an interesting mechanism by which these variants contribute to disease, but this hypothesis requires further work to understand the interactions of tau and α-synuclein, given that the latter is the most pathologically relevant species. It is reasonable to think that α-synuclein risk alleles might increase expression of that protein, especially as multiplication mutations around the *SNCA* locus are causal for PD, but this remains to be proven.

In total, these data show that for genes that change risk of PD over a lifetime, the effects are probably subtle and may in some cases be related to altered mRNA or protein expression levels.

However, there are additional important questions that need to be resolved. Both α-synuclein and tau are expressed in all neurons and yet show association with PD where there is preferential vulnerability of dopamine neurons. This is not an absolutely selective effect as a-synuclein can accumulate in other brain areas (eg the cortex in diffuse Lewy body disease) and tau is associated with frontotemporal dementia, the association with parkinsonism is still striking. LRRK2 expression is actually higher in areas that are targeted by nigral neurons than in the ventral midbrain itself {Galter, 2006 #120} at least at the mRNA level, and thus selectivity here shows an inverse correlation with where the gene is expressed. As discussed for recessive parkinsonism, the reasons for selectivity are not immediately obvious for any of the dominant and risk factor genes. One might speculate that some of the same factors (ROS generation from mitochondrial metabolism) might be involved, but this is extremely speculative. Clearly, understanding why gene mutations or expression differences results in PD is a critical question for the future.

Summary

The rapid pace of discovery in the genetics of PD has lead to a huge amount of data to sort through that will present a challenge for biological understanding over the next few years. Two of the key ideas enunciated here are that understanding how multiple different mutations in the same gene cause disease and, by extension, how multiple genes for the same phenotype work is critical for developing a general pathogenic framework for PD. Importantly, at least some of the genetic influences on PD are shared between rare familial cases and sporadic disease making it feasible to suppose that pathogenic events may be shared between the two sets of etiologies. This in turn suggests that a further understanding of genetic effects might be helpful in developing new ideas about the pathogenesis of PD and eventually for the treatment of this disorder. Finally, the reasons for the preferential effects of mutations in widely expressed proteins on dopamine neurons remain difficult to identify. This, along with the strong effects of aging on PD and related phenotypes, remains a critical next step for the field in trying to understand the pathophysiology of PD.

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Abbreviations

COR	C-terminal of ROC domain
DLBD	diffuse Lewy body disease
GWAS	genome wide association study
LRRK2	leucine rich repeat kinase 2
MAPT	microtubule associated protein tau
PINK1	PTEN induced novel kinase 1
PD	Parkinson's disease
SNP	single nucleotide polymorphism
SNCA	synuclein alpha (gene name)
ROC	Ras of complex proteins domain