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Swiss Cheese et alii, some of the first neurodegenerative mutants isolated in *Drosophila*

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Abstract

Drosophila has only recently become a model organism to study progressive neurodegeneration, mainly using transgenic flies expressing human disease genes. However, classical forward genetics isolating and characterizing fly mutants that show characteristic features of progressive neurodegeneration can also provide a useful tool to get insights into the mechanisms of neurodegeneration. Interestingly, the first such mutants have been already isolated in the 1970s, and this review focuses on the description of four such mutants originally isolated by Martin Heisenberg.

Until this day most of the research done on the adult brain of *Drosophila melanogaster* can be traced back, directly or indirectly, to the founder of neurogenetics Seymour Benzer. So it comes as no surprise that the first neurodegenerative mutant *drop dead* (*drd*) had been isolated in Benzer's laboratory (Hotta and Benzer, 1972). Using a countercurrent apparatus to screen for mutants defective in fast phototaxis (Benzer, 1967), *drd* was one of the first mutants isolated on the basis of its defective behavior. Histologically *drd* displayed a general vacuolization within the brain neuropil and years later when I joined Seymour's group, we found that this was due to a defect in the architecture of the tracheal system in the brain and subsequent hypoxia.

Inspired by his long time friend, Martin Heisenberg started his own hunt for mutants affecting the function of the adult brain (Heisenberg, 1971). Using a Y-maze with rotating "barber poles", he isolated the first mutants that displayed reduced optomotor responses during locomotion (for example *optomotor blind* (*omb*); Heisenberg and Gotz, 1975; Heisenberg et al., 1978)). Behavioral defects were subsequently also used to identify mutants with structural brain defects, based on the rationale that such mutants should be impaired in their locomotion, vision, or coordination. Using the same fast phototaxis assay described by Benzer as a prescreen and a newly developed method for performing mass histology (Heisenberg and Bohl, 1979), they isolated more than 60 anatomical brain mutants; among them the second known neurodegenerative mutant later called *Vacuolar Medulla* (*Vam*; (Coombe and Heisenberg, 1986)). During the following years, generation of students, me being one of them, were involved in isolating more "brain mutants", as part of their basic course in genetics. While the central focus of these efforts was to find structural brain mutants affecting discrete modules of the fly's behavior, also more neurodegenerative mutants were isolated, namely *swiss cheese*, *omb-like*, and *vacuolar peduncle*. Having started to work on neurodegenerative mutants, I remembered those mutants and asked whether I could include them in my studies. To my great pleasure Martin Heisenberg agreed and the cloning and characterization of these mutants has provided the foundation for my research on progressive neurodegeneration in *Drosophila*. Therefore, this review will focus on a short description of the four mutants mentioned above.

swiss cheese (sws)

One of the neurodegenerative mutants isolated in these screens was *sws*, for which five different alleles were isolated. Using different deficiencies the *sws* gene could be mapped to a small interval of approximately 25kb at the chromosomal position 7D1 (Kretzschmar et al., 1997). Subsequent sequencing of the mutant alleles showed that in two of them the mutation is caused by a single amino acid substitution while in a third, *sws^L*, a missense mutation results in a protein of about a quarter of its original size (the mutations in the two remaining alleles could not be identified). *sws* mutant flies develop normal and the central nervous system appears intact in newly eclosed flies at the light microscopic level. However, after five to six days these flies develop vacuoles which become increasingly abundant with further aging (Fig. 1A). The vacuolization is followed by neuronal apoptosis, and premature death of the flies. In addition, glial cells form aberrant membranous structures which could be identified on the electron microscopic level as multi-layered glial wrappings around neuronal cell bodies and axons, already in late pupal stages (Kretzschmar et al., 1997). These glial whorls are reminiscent to the so-called tomaculae seen in patients with hereditary neuropathy with liability to pressure palsies (HNPP) that are caused by swelling of the myelin sheaths (Verhagen et al., 1993).

The SWS protein is a transmembrane protein that has orthologues in many species, ranging from yeast to human (Lush et al., 1998). The human orthologue, called Neuropathy Target Esterase (NTE), has been shown to be a key factor in a neurodegenerative syndrome caused by intoxication with organophosphates, which are components of pesticides and nerve agents (Johnson, 1990; Lotti, 1992; Glynn, 2000; Moretto, 2000). Recently, it has also been shown that mutations in NTE cause a hereditary spastic paraplegia in humans, now called NTE-related Motor Neuron Disorder (Rainier et al., 2008). This disease, which starts in childhood, is characterized by a progressive spastic weakness in the lower extremities and interestingly one of the families afflicted with this condition was found to carry a mutation that changes a methionine conserved in *Drosophila* SWS (Rainier et al., 2008). Mice that lack NTE show severe growth retardation and typically die around day 9 of embryonic development, due to placental failure and defects in vasculogenesis (Moser et al., 2004). However, a tissue-specific knock-out mouse that only eliminated NTE from the nervous system survives through development and reveals a remarkably similar degenerative phenotype as *sws* (Akassoglou et al., 2004). Although these mice show a normal brain structure at two weeks of age, they develop vacuoles in the thalamus and hippocampus, and show aberrant multilayered membranes within the brain when aged. These similarities in phenotypes when SWS/NTE is missing suggested a conserved function of these proteins which was confirmed by the result that mouse NTE can completely replace fly SWS in *sws* mutant flies (Muhlig-Versen et al., 2005). Moreover, these experiments showed that SWS acts cell autonomously because vacuolization of the neuropil can only be rescued by neuronal expression while the glial hyperwrapping is rescued by expression of SWS in glia.

Both fly SWS and mouse NTE are widely expressed in the nervous system, but interestingly their pattern of expression becomes more restricted to large neurons in older animals (Moser et al., 2000; Muhlig-Versen et al., 2005). Furthermore, they both co-localize with markers for the endoplasmic reticulum (ER) and NTE transfected into COS cells is inserted into ER membranes, with most of the protein exposed to the cytoplasmic face (Li et al., 2003). In addition, they contain a highly conserved domain, including a serine which is necessary for the esterase activity of these proteins (Fig. 1B) and which is the binding site for organophosphates (Glynn, 2000). In cell culture, NTE has been shown to degrade ER-associated phosphatidylcholine (PtdCho) to glycerophosphocholine and fatty acids (Zaccheo et al., 2004) and *sws* mutant flies show an increase in PtdCho (Muhlig-Versen et al., 2005). These results suggest that SWS/NTE could act as a brain-specific phospholipase.

PtdCho which makes up to 40% of the phospholipids in eukaryotic membranes (Klein, 2000) is a major component of all cell membranes. A breakdown of PtdCho has been observed in many acute and chronic neurodegenerative diseases and defects in PtdCho metabolism have been associated with growth arrest and apoptosis (Klein, 2000; Du et al., 2003). Increased levels of PtdCho have been connected with Alzheimer's disease (Soderberg et al., 1992) and amyloid β , the major component of amyloid plaques, can increase PtdCho synthesis (Koudinova et al., 2000). In the future, *sws* could therefore provide a unique experimental means to unravel the role of PtdCho homeostasis in neurodegenerative syndromes.

optomotor-blind-like (*olk*)

This mutant was originally named due to its similarity to *optomotor-blind* (*omb*) because the giant fibers of the lobula plate, which are missing in *omb*, were also not detectable in silver stained sections from *olk* flies. In wild type flies reduced silver staining reveals a characteristic network of axonal bundles and fiber tracts which is much less prominent in *olk* mutants. Three different alleles (*olk*¹⁻³) with this phenotype were isolated and mapped to the 1E-2B chromosomal region. A subsequent complementation analysis revealed that *olk* is allelic to *futsch* and Western Blots showed that the expression of the FUTSCH protein is dramatically reduced in *olk*² and *olk*³ and not detectable in *olk*¹ (Bettencourt da Cruz et al., 2005). Genomic sequencing of *olk*¹ showed that it is caused by a missense mutation that shortens the protein to about one third of its original size (probably missing the antibody epitope) which, however, still contains the highly conserved N-terminus. Because the N-terminus has been shown to retain a partial function (Roos et al., 2000), all three *olk* alleles appear to be hypomorphic alleles of *futsch*. Loss of function *futsch* alleles have been shown to result in defects in axonal and dendritic growth in the embryonic nervous system and are embryonic lethal (Roos et al., 2000).

The *futsch* gene encodes a protein homologous to vertebrate MAP1B, a member of the family of microtubule-associated proteins (MAPs; (Matus, 1991; Schoenfeld and Obar, 1994)). MAPs have been linked to a variety of neurological disorders and in particular one member, the tau protein, has been implicated in a whole group of neurodegenerative diseases which have therefore been called tauopathies (Heutink, 2000; Hutton et al., 2001; Lee et al., 2001; Johnson and Bailey, 2002). Probably the best known of these diseases is Alzheimer's Disease, which is characterized by the formation of filamentous inclusions composed of hyperphosphorylated tau called neurofibrillary tangles (Iqbal and Grundke-Iqbal, 2005). Like tau, MAP1B is only expressed in neurons (Matus, 1991) and although MAP1B has not been connected directly with a neurodegenerative disease in humans, it has been shown to accumulate in axons of degenerating Purkinji cells and it was found to associate with autophagosomes in dying neurons (Jackowski et al., 2000; Yue, 2007).

The *futsch*^{*olk*} alleles in *Drosophila* show that also mutations in this MAP can cause progressive neurodegeneration (Bettencourt da Cruz et al., 2005). The degeneration is restricted to the olfactory system and is especially prominent in the mechanosensory neuropil (Fig. 2A) and the projection neurons. The degeneration is not detectable in young flies but becomes apparent after 10d of aging and increases in severity with further aging. Concomitant with the histological phenotype these flies show defects in classical conditioning as revealed by an associative olfactory learning paradigm (Fig. 2B). Given the widespread expression of Futsch in the nervous system (Roos et al., 2000), this specificity for the olfactory system is quite surprising and indeed defects in the microtubule network were found in many or even all parts of the brain in *futsch*^{*olk*}. This suggests that the affected neurons might be especially sensitive to disruptions in the cytoskeleton. In addition, in some neurons redundancy with other MAPs could modulate the deleterious effects and expression of tau in projection neurons can indeed partially suppress the phenotype. Interestingly, *futsch* has also been shown to interact with the

Drosophila homologue of *Fragile × mental retardation*. Mutations in this gene not only suppressed the embryonic phenotypes of the null allele (Zhang et al., 2001) but also delayed the degeneration in *olk* (Bettencourt da Cruz et al., 2005).

These results show that besides tau changes in MAP1B can also result in progressive neurodegeneration. Neurons extend and maintain an elaborate network of axonal and dendritic processes and require intracellular transport over relatively long distances. Integrity of the cytoskeleton is therefore a prerequisite for function and survival of neurons, and although changes in specific cytoskeletal components have been described in many neurodegenerative diseases (reviews see, (McMurray, 2000; Brandt, 2001)) it remains to be seen whether mutations in other microtubule-associated proteins besides tau are leading to progressive neurodegeneration in humans.

vacuolar peduncule (vap)

In addition to the original *vap*^{KS67} allele (now also referred to as *vap*¹), two other alleles have later been isolated in a P-element screen (Botella et al., 2003). *vap* encodes a RasGAP protein and while the mutations in *vap*¹ and *vap*² both generate stop codons within the open reading frame, *vap*³ is caused by an insertion of the P-element into the first intron upstream of the coding region of the gene. Western Blots using an antibody against RasGAP revealed that *vap*³ affects the expression level while *vap*² encodes a shortened protein missing the GAP catalytic domain. *vap*¹, although theoretically resulting in a protein slightly larger than the one encoded by *vap*², appears to be a null allele because no protein could be detected in Western Blots.

Like the mutants described above, *vap* flies show age-dependent neurodegeneration detectable by the formation of vacuoles in the central brain and optic lobes (Fig. 3A; depending on the genetic background, the vacuoles are more prominent along the pedunculi, hence the name; (deBelle and Heisenberg 1996)). In contrast to *sws*, where neurons undergo apoptosis, neurons in the *vap* brain show signs of autophagic cell death, including the accumulation of autolysosomes within the neurons (Botella et al., 2003). Interestingly, this autophagic degeneration resembles the type of cell death found in human cancer cells expressing an oncogenic form of Ras (Chi et al., 1999; Kitanaka and Kuchino, 1999). Because GAP proteins act as direct negative regulators of Ras signaling (Fig. 3B), this suggests that extensive Ras activity due to the loss of RasGAP is causing the cell death in *vap*.

In accordance with this hypothesis, Ras-dependent signaling like MAPK activation was shown to be upregulated in *vap* (Botella et al., 2003). Furthermore, genetic interaction tests with various members of the EGFR/Ras pathway showed that manipulations that further stimulated activation of Ras enhanced the degenerative phenotype in *vap* whereas manipulations that resulted in an inhibition of Ras suppressed the phenotype. These results confirmed that the neurodegenerative phenotype in *vap* flies is indeed due to an aberrant regulation of the Egfr/Ras signal transduction cascade. Interestingly, however, expression of other GAP proteins did not have beneficial effects because neither GAP1 nor NF1 were able to rescue the mutant phenotype when expressed in neurons of *vap* mutants. This suggests that RasGAP has a specific function in neuronal survival which may be conserved in mammals because mice lacking RasGAP also show extensive neuronal cell death (Henkemeyer et al., 1995). In addition, the *vap* mutant provides for the first time evidence that EGF receptor activity is essential for the survival of neurons in *Drosophila*, suggesting that its putative ligand in the adult brain acts like a neuronal growth factor. This is especially intriguing, because no neurotrophic factor could be identified in *Drosophila* so far.

Vacuolar medulla (Vam)

For *Vam*, only a single allele (*Vam*^{KS74}) has been isolated and interestingly this was the only mutation resulting in a semi-dominant phenotype. While hemizygous males and homozygous females show first vacuoles already one hour after eclosion, heterozygous females have to be aged for five to six days before this phenotype becomes apparent. As mentioned above, *Vam* was one of the first mutants which showed progressive degeneration and is still one of the few mutants that selectively affect only a few specific neurons. *Vam* is characterized by the formation of vacuoles which have originally been described to form mainly in the distal medulla, the second optic ganglia, commencing at eclosion (Coombe and Heisenberg, 1986). Although the vacuolization is still restricted to the medulla, we now detect vacuoles in all areas of the medulla (Fig. 4A). The spatial restriction to the medulla appears to be due to the degeneration of the lamina monopolar neurons L1 and L2 which have their synaptic target area in the distal medulla. The dying neurons reveal the characteristic signs of apoptotic cell death, including nuclear fragmentation and membrane blebbing. Supporting apoptosis of these neurons, TUNEL positive cells can be detected in the lamina cortex, where the cell bodies of these monopolar cells are localized (Tschape et al., 2003).

This histological phenotype is accompanied by behavioral defects (Coombe and Heisenberg, 1986); even one day old flies neither show a landing response nor do they react to vertically moving stripes. In addition, they reveal severe deficits in optomotor response and are impaired in fixating moving objects during walking or flying. The loss of motion detection in several assays suggested that the degeneration of the monopolar cells in *Vam* affected the visual input these flies receive. This was verified by electroretinograms which showed that the on and off transients generated by the lamina are missing while the photoreceptor potential was not significantly different from wild type (Fig. 4B).

It is noteworthy that most of the identified human genes that are involved in neurodegenerative diseases are also inducing dominant phenotypes, for example Huntingtin (Huntingtons Disease) or Amyloid Precursor Protein and Presenilin (Alzheimer Disease). The dominant effect of *Vam*, together with its specificity for only two types of monopolar cells, make this mutant one of the most intriguing mutants identified in these screens. Unfortunately, however, the gene could not be identified yet but hopefully the molecular characterization of the VAM protein will eventually provide insights into the function of this interesting protein.

Perspectives

When these mutants were isolated three decades ago, only a few people thought it possible that *Drosophila* could be used to investigate the mechanisms of progressive neurodegeneration. In recent years, however, *Drosophila* has been increasingly accepted as a valuable model system for identifying genes and pathways that contribute to neurodegenerative diseases in humans (reviews see; (Bonini and Fortini, 2003; O'Kane, 2003; Shulman et al., 2003; Botas, 2007)). Luckily, Martin Heisenberg had the foresight to maintain the original mutant lines, which have proven to be a valuable resource for investigating the genetic basis of neurodegeneration. As life expectancies continue to increase, neurodegenerative diseases pose a progressively more severe threat to our health, and the interest in this field will certainly not degenerate. Although most research groups have focused on transgenic fly models that express human genes associated with a particular neurological condition (for example; (Jackson et al., 1998; Warrick et al., 1998; Feany and Bender, 2000; Greeve et al., 2004)), classical forward genetic screens continue to provide a powerful, unbiased method for identifying genes that are involved in neurodegeneration. More recent screens of this type have identified a variety of *Drosophila* mutants that exhibit different patterns of neurodegeneration (Min and Benzer, 1997; Palladino et al., 2002), while suppressor and enhancer screens using available mutant lines can now be employed to identify pathways that modulate their phenotypes. Inevitably, it must be noted

that not all “neurodegenerative” mechanisms in flies may be absolutely conserved in humans (and vice versa); nevertheless, characterization of many of these genes at the molecular, cellular, and whole-animal level has revealed a surprising degree of conservation in the basic mechanisms that maintain the health of the adult brain in both flies and humans. Clearly, flies rely on an intact brain as much as humans do, suggesting that this model systems approach will continue to provide important insight into the mechanisms of neurodegeneration in the coming years.

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Figure 1.

sws shows progressive degeneration and lacks esterase activity. A) A 14d old *sws¹* mutant shows vacuolization in all brain regions. (B) Wild type flies reveal NTE-like esterase activity similar to the activity measured in mouse brain. This NTE-like activity is reduced in heterozygous *sws¹* flies and reduced to the baseline of the assay in homozygous *sws¹* and *sws⁴* mutants. Re=retina, La=lamina, Me=medulla, Lo=lobula, Lopl=lobula plate, Al=antennal lobe. Scale bar=50μm.

Figure 2.

olk interferes with the anatomy and function of the olfactory system. A) A 20d old *olk¹* fly shows a large vacuole in the mechanosensory neuropil of the antennal lobe (arrow). B) Already in 5d old *olk¹* and *olk³* flies the associative olfactory learning is severely impaired compared to wild type of the same age. Re=retina, La=lamina, Me=medulla, Lo=lobula, Lopl=lobula plate, Al=antennal lobe. Scale bar=50μm.

Figure 3.

vap, a RasGAP protein, causes neurodegeneration by aberrant activation of the Egfr/Ras pathway. A) A 7d old *vap¹* fly shows several vacuoles in the medulla (arrows). B) *vap* encodes a RasGAP protein, which facilitates the transition of activated GTP-bound ras to inactive GDP-bound ras. Re=retina, La=lamina, Me=medulla, Lo=lobula, Lopl=lobula plate. Scale bar=50 μ m.

Figure 4.

Vam specifically affects monopolar cells and causes an aberrant ERG. A) Vacuoles are detected in the medulla, the target field of monopolar cells, of a 5d old *Vam* mutant (arrows). B) Already in young *Vam* flies (1d) the prominent on and off transients seen in the ERG of wild type flies is missing while the photoreceptor potential appears unaltered (modified after Coombe and Heisenberg, 1986). Re=retina, La=lamina, Me=medulla, Lo=lobula, Lopl=lobula plate. Scale bar=50 μ m.