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Phenotyping animal models of diabetic neuropathy: a consensus statement of the diabetic neuropathy study group of the EASD (Neurodiab)

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The Working Group of Neurodials

Abstract

NIDDK, JDRF, and the Diabetic Neuropathy Study Group of EASD sponsored a meeting to explore the current status of animal models of diabetic peripheral neuropathy. The goal of the workshop was to develop a set of consensus criteria for the phenotyping of rodent models of diabetic neuropathy. The discussion was divided into five areas: (1) status of commonly used rodent models of diabetes, (2) nerve structure, (3) electrophysiological assessments of nerve function, (4) behavioral assessments of nerve function, and (5) the role of biomarkers in disease phenotyping. Participants discussed the current understanding of each area, gold standards (if applicable) for assessments of function, improvements of existing techniques, and utility of known and exploratory biomarkers. The research opportunities in each area were outlined, providing a possible roadmap for future studies. The meeting concluded with a discussion on the merits and limitations of a unified approach to phenotyping rodent models of diabetic neuropathy and a consensus formed on the definition of the minimum criteria required for establishing the presence of the disease. A neuropathy phenotype in rodents was defined as the presence of statistically different values between diabetic and control animals in 2 of 3 assessments (nocifensive behavior, nerve conduction velocities, or nerve structure). The participants propose that this framework would allow different research groups to compare and share data, with an emphasis on data targeted toward the therapeutic efficacy of drug interventions.

Keywords

diabetes; diabetic neuropathy; neuropathy; peripheral neuropathy

Introduction

This workshop was chaired by Professors Mary Cotter (University of Aberdeen, Scotland) and Eva Feldman (University of Michigan, USA), who charged the invited attendees to

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explore the current definition of neuropathy in diabetic rodents and develop a uniform set of criteria for establishing the presence of neuropathy in these models. The goal of the workshop was to form a dialog that will lead to an improved consensus in the field of animal models of diabetic neuropathy and an accelerated approach to both basic and translational research. The attendees were also encouraged to identify major scientific gaps in the current approach of defining animal models of diabetic neuropathy with the intent of identifying new approaches and research opportunities.

Initial presentations reviewed the current understanding of animal models of diabetic neuropathy and known assessments for the presence of somatic neuropathy in rodent models. The workshop then explored new research areas in neuropathic pain, as well as new biomarkers and technologies for the diagnosis of neuropathy. The need to adopt a uniform set of phenotypic criteria for diabetic neuropathy in rodents was highlighted at the conclusion of the workshop and a working set of criteria was proposed.

In this meeting summary, after an introduction into the status of known rodent models, four areas of interest are discussed separately: peripheral nerve structure, electrophysiological assessments of nerve function, behavioral assessments of nerve function, and the role of biomarkers in disease phenotyping. The meeting focused on somatic nerves, although the importance of diabetic autonomic neuropathy was also recognized and the current paucity of experimental data lamented. Suggestions pertinent to this area are therefore interpolated throughout this summary. Within each area of interest, an introduction to the basic tenets of the area is followed by a section highlighting interesting controversies in the field and a list of new ideas that require additional basic and translational investigation to enhance the field. This report has also been supplemented with citations that are intended to assist the reader by either providing an overview to a broad area of discussion or to highlight a very specific point. The literature cited was not specifically identified during the meeting and the intent is not to provide a comprehensive literature review.

Diabetic neuropathy

Diabetic neuropathy is one of the leading causes of disability in the Western world and is associated with significant morbidity and cost to society. In man, neuropathy is characterized by symptoms and signs that reflect structural damage to the peripheral nervous system and the accompanying dysfunction of nerves and the organs they supply (Tesfaye et al., 2010). Despite the prevalence of diabetic neuropathy and its toll on society, a basic understanding of disease mechanisms remains incomplete. In parallel, treatment of diabetic neuropathy is largely restricted to emphasizing tight glucose control, with more targeted therapies being approved only in a few countries worldwide. The limited therapeutic options available to physicians and their patients highlights the need for animal models of diabetic neuropathy that accurately represent the human condition to facilitate development and testing of disease-based, mechanistic therapeutics.

Establishing criteria that define neuropathy in diabetic rodents is an essential component to the development and use of animal models to advance our understanding of the pathogenesis and treatment of diabetic neuropathy. To accomplish this goal, attendees of the workshop first reviewed the animal models currently used to study diabetic neuropathy.

Rodent models of diabetic neuropathy

Animal models can provide a valuable resource in the study of diabetic neuropathy. It is essential to be aware of the systemic physiologic conditions (hyperglycemia, insulin deficiency, insulin resistance, dyslipidemia, blood pressure) that are present in each model of diabetes.

Diabetic neuropathy is a dynamic and progressive disease that, in animal models, begins with an *acute metabolic phase* featuring nerve conduction velocity slowing and hyperalgesia that are usually reversible. As the duration of diabetes progresses, more severe functional abnormalities develop in concert with onset of progressive structural changes in the nerve. This *chronic structural phase* is less amenable to metabolic interventions. It is not entirely clear that the mechanisms underlying these phases share a common pathogenesis and the absence of complete understanding of the pathogenic cascade that produces the multiple manifestations of neuropathy highlights the importance of establishing and defining clear criteria for models of diabetic neuropathy in rodents.

The streptozotocin diabetic rat—The streptozotocin (STZ) diabetic rat has, to date, been the most commonly used model of diabetic neuropathy. Diabetes is induced by a single intraperitoneal or intravenous dose of 40–80 mg STZ/kg body weight, depending on such factors as total body weight and severity of anticipated disease. Diabetes is usually defined by blood glucose concentrations of 15 mM/l (270 mg/dl) or greater. The minimum duration of diabetes depends upon the aspect of neuropathy under investigation and can be as little as 2–4 weeks for features such as allodynia and nerve conduction velocity slowing while substantially longer durations (at least 8–16 weeks) are required to expose some structural features of neuropathy. Although high doses of STZ can induce acute nephrotoxicity in rodents, there is substantial evidence arguing against the suggestion that STZ toxicity is directly responsible for the neuropathy phenotype. For example, the progressively developing neuropathy of STZ diabetic rats is not characteristic of acute neurotoxicity and many nerve disorders that develop in STZ diabetic rodents can be reversed after specific therapeutic interventions. Animals treated concurrently with both STZ and O-methyl-glucose, an agent that blocks the effects of STZ on the pancreas, show no evidence of neuropathy (Davidson et al., 2009).

There are clear advantages to using the STZ diabetic rat model. It is relatively inexpensive and there is considerable published data available. The STZ diabetic rat demonstrates robust early behavioral and electro-physiological changes with mild, but quantifiable, later changes to nerve structure. There are also clear limitations, most notably the lack of robust nerve pathologies such as demyelination and fiber loss in nerve trunks, except after years of disease. From a practical viewpoint, STZ diabetic rats may lose weight and become cachectic unless given regular low dose insulin to allow long-term maintenance.

The BB/Wor and BBZDR/Wor-rat—Diabetes prone BB/Wor rats lack T lymphocytes that express the RT6 alloantigen, precipitating auto-immune attack of the pancreas (Yang and Santamaria, 2006). The BB/Wor-rat develops spontaneous onset of type 1 diabetes in males between the ages of 70–80 days while diabetes resistant BB/Wor rats provide

appropriate controls. Diabetes prone BB/Wor rats have complete insulin/C-peptide deficiencies and require daily insulin supplementation. The BB/Wor-rat develops nerve conduction changes after 2 weeks of diabetes and there are reports of sural nerve fiber loss and structural pathology by 4 months. These animals can be maintained for over a year, with the level of hyperglycemia adjusted by titrating insulin delivery. The key advantage of the BB/Wor-rat is that the primary disease closely reflects type 1 diabetes. However, these animals are expensive and very labor intensive to maintain.

The BBZDR/Wor rat was derived from crossing diabetes resistant BB/Wor rats with the Zucker rat, which has defective leptin receptors and is thus insulin resistant (Tirabassi et al., 2004). Similar to its type 1 counterpart, the BBZDR/Wor-rat spontaneously develops diabetes in males 70–80 days of age. However, the onset of diabetes is preceded by obesity and these animals model type 2 diabetes. The BBZDR/Wor-rat develops insulin resistance, hyperinsulinemia, hyperglycemia, hypercholesterolemia, dyslipidemia, and hypertension late in the disease course. Conduction velocity deficits and thermal hyperalgesia have been identified within 4 weeks of diabetes. Structural changes, including sural nerve fiber loss, differ qualitatively from those in type 1 BB/Wor-rat (Sima et al., 2000). The major advantage of the BBZDR/Wor-rat is that these animals spontaneously develop hyperglycemia and can be maintained for a long period of time. Disadvantages include cost and the fact the rats become increasingly ill, requiring insulin supplementation.

Rat models of impaired glucose tolerance—Zucker (*fa*/*fa*) and Zucker Diabetic Fatty (ZDF) rats exhibit leptin receptor deficiency, promoting hyperphagia (Fellmann et al., 2013). Both strains initially develop impaired glucose tolerance but only the ZDF progresses to overt diabetes at 8–10 weeks of age, because of impaired pancreatic beta cell function. As animals progress through the stages of pre-diabetes (Zucker) to frank diabetes (ZDF), there are parallel increases in hyperinsulinemia, hyperlipidemia and, later in the disease course, hypertension. Nerve conduction velocity slowing progresses with time, mechanical allodynia develops, and there are reports of IENF loss. The primary disadvantages of these models are cost, the need for close attention to sexual organ hygiene in males and high mortality rates after 4–6 months of diabetes. A separate rat model of impaired glucose tolerance, the GK Stockholm rat, develops moderate obesity, insulin resistance, moderate hyperinsulinemia, and hypertension. This model shows early small fiber deficits and nerve conduction velocity deficits develop late in the course of disease. Similar to the ZDF rat, these animals are expensive and die early.

Diabetes in mice (Sullivan et al., 2007)—One clear incentive for phenotyping neuropathy in the mouse is that diabetes can be induced in the wide range of existing mice that have undergone selective genetic manipulations to highlight specific pathogenic mechanisms and disease targets. A disadvantage with any mouse model is its size, which can preclude certain experimental techniques.

Diabetes can be induced in both inbred and out-bred mouse strains by intraperitoneal or intravenous STZ, administered either as one high dose in the 150–200 mg/kg range, or as lower doses given over consecutive days such as 90 + 90, 85 + 70 + 55, or 100 + 40 + 40 mg/kg. The Swiss-Webster (outbred), C57/Bl6 and CD1 (both inbred) mouse strains

currently represent the best-characterized models. Nerve conduction velocity slowing, impaired responses in sensory tests, and IENF loss occur within 2–8 weeks of diabetes and there are reports of distal nerve fiber loss, axonal atrophy and myelin thinning after many months of diabetes. STZ diabetic mice can revert to normoglycemia, presumably due to proliferation of residual pancreatic beta cells, and there can also be high mortality rates in the absence of insulin supplementation. Outbred strains of mice are not used for transgenic studies.

There are two common genetic mouse models of type 1 diabetes: the non-obese diabetic (NOD) mouse and the Akita mouse, representing spontaneous auto-immune type 1 diabetes (NOD) and a mutation of the insulin-2 gene, respectively. There is currently relatively little consistent information on the neuropathy in these animal models. The Akita model on the C57Bl6/J background has been reported to develop anything between mild and severe neuropathy depending on the investigating laboratory and assays used. The Akita mutation is being placed on several different inbred background strains with the goal of producing improved models of neuropathy. These animals are costly and difficult to maintain, as they frequently require daily insulin supplementation.

Models of type 2 diabetes, such as db/db (leptin receptor mutation) and ob/ob (leptin mutation) mice, have been available for many years and are being increasingly used by investigators interested in diabetic neuropathy. Both models are available on the C57 BKLS and Bl/6 J backgrounds and develop diabetes at 4–6 weeks of age with hyperinsulinemia and hyperlipidemia. Nerve conduction velocity slowing and behavioral deficits are established with 4–8 weeks of diabetes and progress over time. These animals can exhibit IENF loss, and structural abnormalities of the sural nerve are also reported at late stages of the disease. The main limitation of both the db/db and ob/ob models is that death occurs at 24–30 weeks of diabetes in the absence of insulin supplementation. There is a growing appreciation among investigators that the phenotype of both diabetes and somatic neuropathy may be background specific.

Pre-diabetes/metabolic syndrome—There is increasing interest in the use of high fat diets to model aspects of pre-diabetes and metabolic syndrome such as insulin resistance and dyslipidemia that may contribute to the pathogenesis of diabetic neuropathy independent of frank hyperglycemia. Rodents fed a high fat diet become obese but have relatively normal life spans. Both rats and mice fed diets containing 45–60 kcal% fat have been reported to develop aspects of peripheral neuropathy such as nerve conduction slowing, altered behavioral responses to sensory stimuli, and epidermal fiber depletion.

Peripheral Nerve Structure

Background

The pathology of human diabetic neuropathy has been largely defined by microscopic examination of thick and thin sections and of teased fibers, while morphometric analysis is used to quantify the type and extent of damage. Structural nerve injury in human diabetic neuropathy is well documented in both the somatic and autonomic components of the peripheral nervous system. Less information on diabetes-induced changes in the brain is

available, although this is a current focus (Selvarajah et al., 2011) and may have parallels with other neurodegenerative diseases. In the peripheral nervous system, structural changes have been documented in sensory and motor terminals and in somatic and autonomic ganglia and nerve trunks. Reactive, degenerative, and reparative responses of neurons, axons, terminals, and Schwann cells are evident in both myelinated and unmyelinated fibers. Although not specific to diabetes-induced nerve injury, the pathologies represent the repertoire of cellular responses to a variety of metabolic, toxic, immunologic, and physical injuries.

Nerve injury in human diabetic polyneuropathy presents as a “stocking and glove” neuropathy with dying back of peripheral terminals and distal nerves (Tesfaye et al., 2010). This can be measured in skin biopsies by loss of IENF and in nerve trunk biopsies as damage to, and ultimately loss of, both unmyelinated and myelinated fibers (Malik et al., 2011). Axonal dystrophy also occurs in both unmyelinated and myelinated fibers, overwhelming the capacity of axon-Schwann-cell networks to remove this debris. Subsequent Wallerian degeneration of myelinated fibers leads to the collapse of the axon with secondary myelin loss. Reparative responses, indicated by regenerative clusters, occur but ultimately are inadequate, resulting in the fiber loss observed in many nerve biopsy studies. Schwann cells are often an independent site of injury and accumulate glycogen, lysosomes, and lipid droplets, as well as enlarged mitochondria with effaced cristae. Subsequently, primary demyelination at both internodal and paranodal sites occurs and is followed by remyelination, indicated by axons with disproportionately thin myelin sheaths often surrounded by supernumerary Schwann cell processes. Both primary and secondary demyelination has been documented in the same biopsy, indicating that diabetes targets both Schwann cells and axons (Behse et al., 1977).

The vast majority of studies assessing nerve structural injury are restricted to two models of type 1 diabetes, STZ diabetic rats, and BB/Wor rats, with relatively few studies appearing to date in models of type 2 diabetes. With the interesting exception of spontaneously diabetic cats (Mizisin et al., 2007), appropriately fixed and prepared nerve trunks do not exhibit the dramatic fiber loss and demyelination documented in human diabetic neuropathy. However, recent studies quantifying depletion of IENF and corneal nerves and description of neuroaxonal dystrophy in prevertebral sympathetic ganglia and myenteric nerves are promising, as this pathology appears comparable to that observed in diabetic patients.

Recommendations

- Assessment of neuronal number should always be made with unbiased stereological techniques and quantification of both total fiber number and fascicular area is essential to interpret shifts in size-frequency distribution and changes in fiber density.
- Time-course studies with longer durations of diabetes are recommended as a means of better understanding how structural nerve injury develops and progresses.
- It is clear that a future research focus will be on mice because this species is amenable to spontaneous and targeted mutation of genes that will allow dissection of the pathogenetic processes involved in diabetic neuropathy. However, this

requires that structural nerve injury in mouse models must be better characterized, as presently there is a relative paucity of relevant observations. It is unlikely that a single mouse model will suffice and it will be important to identify common themes across multiple models to ensure that strain-specific issues will not be misinterpreted as basic pathologic findings.

- Studies of models of type 2 diabetes are particularly recommended, as the majority of patients with diabetic neuropathy have this form of the disease (while noting that autopsy-based studies often involve patients in whom secondary beta cell exhaustion with resultant insulin deficiency has eventually been superimposed). In particular, time-course observations that take into account dyslipidemia, hypertension, and the initial hyperinsulinemic and subsequent hypoinsulinemic phases of type 2 diabetes should be undertaken. This may be critical to understanding perceived differences between neuropathy in type 1 and type 2 diabetes. Nerve biopsies from hyper-insulinemic type 2 diabetic patients or those with impaired glucose tolerance will also be necessary to define early changes for comparison with the data from animal models.
- The best currently available structural end point in experimental diabetes that parallels nerve injury in human diabetic neuropathy appears to be IENF density, although depletion of corneal nerves may be a viable surrogate. The impact of pre-diabetes, type 1 and type 2 diabetes on dermal and epidermal innervation warrants further consideration, as does the response to potential therapeutic agents. Given the coherence of epidermal fiber loss in rodent models of diabetes and diabetic patients, plantar nerve ultrastructure should be investigated for distal nerve injury in rodents that is comparable to that described in human nerve biopsies.

Controversies and limitations

Some workshop attendees advocated that more technically challenging techniques, such as teased fiber analysis, should be encouraged to provide an accurate means of identifying axonal and Schwann cell injury in the form of axonal degeneration and demyelination. It was also considered important to recognize that diabetic neuropathy is probably a group of neuropathies with differences in pathology and pathogenesis. It is noteworthy that not all neuropathies in diabetic patients are diabetic neuropathy, as illustrated by the apparent increased incidence of chronic inflammatory demyelinating polyneuropathy (CIDP) in diabetic patients (Knopp and Rajabally, 2012).

Research opportunities

The attendees uniformly agreed that there is a large unmet need in the study of peripheral nerve structure and in the use of structural changes in understanding disease pathology. Several areas were identified that require investigation by the scientific community. These include:

- Characterization of cutaneous innervation needs to move beyond immunostaining with the pan-neuronal marker PGP9.5 to consider the potential impact of diabetes on separate peptidergic and non-peptidergic populations.

- Methods such as the capsaicin model of IENF injury could be developed or adapted to assess the regenerative potential of epidermal innervation in experimental diabetes and the efficacy of potential therapeutic agents at promoting regeneration.
- Observations on the impact of diabetes on other sensory endings in skin, such as Meissner's and Pacinian corpuscles, are limited and need to be developed in terms of quantification of both density and other alterations in morphology that may precede overt terminal loss.
- Studies addressing potential structural alterations in autonomic innervation of cutaneous blood vessels and sweat glands need to be developed, as they are potentially key to the mechanisms leading to foot ulceration.
- Studies investigating the innervation of intestinal mucosa could also be applied to animal models of diabetes and would offer a distal compliment to existing studies on experimental diabetic autonomic neuropathy.
- The occasional clinical and experimental studies documenting the impact of diabetes on motor terminals and their skeletal muscle effectors should be extended.
- The structural and functional inter-relationships of myocardial innervation and the pattern of cardiac autonomic denervation need to be characterized as a basis for understanding the efficacy of therapeutic interventions at promoting cardiac re-innervation.
- Given the recent focus on diabetes-induced changes in the brain and the role of spinal processing in diabetic painful neuropathy, a systematic investigation of the impact of experimental diabetes on spinal cord and brain structure is needed.
- There is no existing animal model that faithfully replicates the structural changes associated with microangiopathy present in diabetic patients. An animal model that combines diabetes, hypertension, and dyslipidemia might fulfill this need.
- Given that angiogenic therapeutic agents are being developed, resolution is needed with regard to whether experimental diabetic neuropathy is associated with changes in endoneurial blood vessel number and/or density.
- Clinical advances in non-invasive imaging by PET, fMRI, corneal confocal microscopy and other techniques may be applicable to animal models of diabetic neuropathy.

Electrophysiology

Background

Electrophysiological assessment of nerve function in diabetic patients provides a quantitative measure of nerve integrity and a means to monitor disease onset and progression (Tesfaye et al., 2010). In rodent models of diabetic neuropathy, somatic nerve conduction velocity measurements are commonly used to define the extent of nerve dysfunction as well as providing an endpoint in studies of putative treatments. Conduction velocity is usually calculated by measuring M and H wave latency from muscle electromyogram (EMG) recordings following nerve stimulation at two sites or, less

commonly, by direct recording of nerve compound action potential latency. When performed with careful attention to detail, nerve conduction studies are both reliable and reproducible and remain a useful measure of nerve dysfunction in animal models of diabetes. However, a number of confounding variables exist which need to be carefully controlled in order to make the measurements as comparable as possible between investigators.

Recommendations

- To help ensure an exact, unbiased assessment of nerve electrophysiology for phenotyping animals with diabetic neuropathy, nerve conduction velocity should be measured from at least one motor and one sensory nerve.
- Nerve temperature affects nerve conduction velocity measurements, varying by approximately 1 m/s/°C. Diabetic rodents have cold extremities, so measurement of core temperature is not sufficient to ensure standardized conditions are met for nerve conduction measurements. It is therefore imperative that near-nerve temperature be monitored and maintained as a constant across groups of animals.
- EMG recordings primarily allow measurement of conduction in the largest myelinated fibers, which do not attain their maximum diameter or velocity until they are fully mature (at least 3–4 months of age in rats) (Moore et al., 1980; Saitua and Alvarez, 1988). It is therefore important that both onset and age-matched measurements are made to allow interpretation of differences between control and diabetic groups. This makes it clear whether any changes can be directly attributed to diabetes or are confounded by stunting of maturation.
- Measurements of caudal nerve conduction velocity should only be used as part of a panel that includes other motor and sensory nerves that are more directly comparable with man.

Controversies and limitations

There was general agreement that guidelines should not restrict potentially novel research, such as studies of autonomic nerve function, where changes in nerve conduction velocity appear to be relatively small. There was not general agreement among workshop attendees if pre-set limits of diabetes-induced nerve conduction velocity change are necessary, or if demonstration of a statistically significant difference is sufficient. Another area of controversy is the utility of muscle EMG amplitudes, which are useful in human studies of diabetic neuropathy. Participants were of the opinion that there is little value in monitoring changes in EMG amplitude in diabetic rodents because results are strongly influenced by muscle wasting and electrode placement or movement, so do not provide reliable and comparable information about nerve function. Nerve action potential amplitudes are potentially informative but not commonly measured.

Large fiber conduction velocity has been the primary endpoint in the majority of clinical trials for drugs to treat diabetic neuropathy and there is a long and ongoing controversy as to whether nerve conduction velocity slowing is an appropriate surrogate for small fiber neuropathy (Chalk et al., 2007; Lauria and Lombardi, 2012). However, it was agreed that,

while nerve conduction velocity will be an important measurement of nerve dysfunction in animal models of diabetes so long as it remains the clinical “gold standard” for drug trials, phenotyping studies and screening of potential therapies for diabetic neuropathy should include other measures of nerve function and structure (see *General recommendations* section). Intervention studies against an existing deficit are likely to be most useful when evaluating potential treatments, as clinical trials are generally designed as interventions against established neuropathy. Nerve conduction velocity changes do not provide a good correlate for regeneration studies because confounding factors, such as internodal distance, preclude restoration of control values, while not reflecting the efficacy of regeneration.

Research opportunities

Multiple studies in man suggest that small fiber neuropathy currently represents the earliest quantifiable injury in diabetic neuropathy. Consequently, new measurements of small fiber function need to be developed. These could include microneurography, monitoring the neurogenic skin reflex to sensory nerve stimulation or utilizing autonomic tests, such as estimation of corpus cavernosal pressure changes after nitrergic nerve stimulation and assessment of alterations in cardiac baroreceptor reflexes and sympathetic nerve activity, as objective measures of neuropathy.

Behavioral Assessment of Nerve Function

Background

Signs and symptoms of sensory dysfunction, such as sensory loss, spontaneous pain, paresthesias, or allodynia, are frequently the earliest and most evident manifestations of diabetic neuropathy to both patients and their physicians (Tesfaye et al., 2010). There are no approved therapies to recover sensory loss, and treatments for painful neuropathy are inconsistent, have frequently debilitating side effects, and do not address the pathogenesis of diabetic neuropathy. There is therefore an urgent need to understand the etiology of diabetic sensory neuropathy and utilize animal models to assess the potential of novel therapeutic approaches.

Strong evidence indicating the presence of spontaneous pain in diabetic rodents is lacking and the experience of the group is that otherwise healthy diabetic rodents do not display pain-associated behaviors such as behavioral depression, audible or ultrasonic vocalizations, limb guarding or autotomy. Investigators have therefore been largely confined to measuring behavioral responses to sensory stimuli as indices of sensory dysfunction in diabetic rodents. This parallels the use of quantitative sensory testing (QST) in diabetic patients to assess peripheral sensory function and subsequent central nervous system processing. There are, however, important differences, including the reliance in rodent studies on measuring aversive responses that imply perception of discomfort or pain, rather than perception of stimulus, as measured in QST. Limb responses in rodents also incorporate motor neuron and effector function. Consequently, behavioral tests commonly employed in diabetic rodents should be presented and interpreted as measures of combined sensory/central nervous system/motor function and not solely as measures of “pain” or “sensory function” unless convincing evidence dictates otherwise.

Recommendations

To help ensure precise, unbiased assessment of behavior as an index of nerve function, the following guidelines are recommended.

Careful choice of behavioral test—Of the many tests applied to diabetic rodents, the majority of published studies use sensory stimuli provided by temperature, pressure, or chemical agents applied to the paw or tail. There is no currently accepted “gold standard” test, and the diverse nature of the sensory systems activated by different stimuli precludes selection of a single assay system. Workshop attendees recommend that investigators avoid limiting their studies to a single test but employ multiple assays to identify disorders in diabetic rodents for mechanistic and drug evaluation studies. There are particular concerns about tests that involve handling of animals during measurements, due to the potential for stress-associated modifications of behavior (Butler and Finn, 2009) and also those that stimulate the tail, due to the unique anatomy of this organ. Consequently the attendees recommend that investigators employ at least one, and preferably a number, of the following commonly used tests:

- Paw withdrawal response latency to escalating heat applied to the plantar surface (Hargreaves method with stimulation of a single paw at any time). *Advantages:* analogous to aspects of QST, no animal restraint required, rate of heating can be varied to stimulate different sensory fiber types. *Caveats*: mechanistic reasons for variable reports of hyperalgesia or hypoalgesia in diabetic rats are unresolved, short inter-test intervals can prompt learning behaviors in rats (but not mice), response times may potentially be influenced by changes in skin thickness and collagen modification.
- Paw withdrawal response to light touch of plantar surface (von Frey monofilaments or automated pressure filaments). *Advantages:* analogous to aspects of QST, no animal restraint required, consistent reports of allodynia in diabetic rats, selective A fiber mediated response. *Caveats*: subjective interpretation of paw withdrawal response, variable methods for applying pressure with von Frey monofilaments, possible differences in stimulus between von Frey monofilaments (focal) and automated (incremental) filaments, current lack of consensus regarding impact of diabetes on this test in mice, with both allodynia and loss of function reported.
- Paw intradermal formalin-evoked responses (0.2–5.0% formalin solution, depending on intensity of stimulus and response required): *Advantages:* no animal restraint required during the response period, consistent reporting of hyperalgesia in diabetic rats and hypoalgesia in diabetic mice, repeatable separation of initial injury (phase 1) and spinally modified (phase 2) components. *Caveats*: no clinically equivalent test, potential subjective identification and interpretation of responses (flinch vs. guarding vs. licking), complex stimulation of multiple sensory fiber types, unknown mechanistic reasons for divergence in response between diabetic rats and mice, restrictions on use of the procedure in some countries.

Other viable, but less widely used, tests that do not require restraint or tail stimulation include:

- Paw response frequency to pinprick of plantar surface.
- Paw response frequency to light brush of dorsal or plantar surface.
- Paw response frequency to cooling.

The following tests are used, but workshop attendees recommend that they be employed only in conjunction with one of more of the tests listed above:

- Paw response to escalating mechanical pressure (Randall–Selitto test). *Caveats:* requires animal restraint, clinical relevance of stimulus unresolved.
- Aversive response to fixed noxious heat (hot plate test). *Caveats:* complex and rapid stimulation of all paws and the tail evoking complex and variable behavioral responses.
- Tail withdrawal from escalating or fixed heat or cold stimuli (tail flick test): *Caveats:* no human anatomical equivalent, spinal reflex may exclude or modify higher CNS processing, may require animal restraint.
- Aversive responses to compression of the tail. *Caveats:* no human anatomical equivalent.

Careful choice of experimental design—Aside from the choice of test, there was general consensus that careful consideration is required of the following design issues to help clarify data interpretation:

- Validate a testing frequency that minimizes modifications of the response due to learning and/or memory.
- Report the time between any last drug treatment and behavioral testing. Where drug treatments are provided over many days, establish the effect of a single drug treatment on the behavioral assay.
- Provide measurements confirming the health status of diabetic animals and their capacity to respond to stimuli. Animals with severe cachexia or showing signs of behavioral withdrawal should be excluded from behavioral tests and the drop out frequency reported. Assays suggesting loss of sensory function should be interpreted with particular caution and accompanied by measurements of function in a performance test such as the rotarod balance test.
- Where possible, record behavioral tests to allow *post hoc* quantification by multiple observers.

Controversies and limitations

It was recognized that the pertinence of aversive behavioral tests to the spontaneous pain of many diabetic patients may be limited and that many caveats should be acknowledged when extrapolating from pain-related behaviors in animals to the human condition (Le Bars et al., 2001). Some members of the group suggested that, because QST and the analogous tests in animals were the least reproducible of the phenotyping components under discussion, they should be included only as adjuncts to any composite phenotyping evaluation.

Research opportunities

A number of research questions remain unanswered and renewed interest in the central processing of pain illustrates the need for new or modified behavioral assays. Ideas for new avenues of investigation include:

- Non-aversive tests such as beam walking and gait analyses should be developed and validated.
- Inter-species variability of the effects of diabetes on certain behavioral tests requires clarification.
- The extent to which measurements interpreted as identifying sensory loss reflect impaired function of the nervous system vs. loss or structural damage to sensory end organs is not known.
- Between-laboratory variations in the impact of diabetes on paw responses to heat require resolution.
- The impact of diabetes on sensory processing in the spinal cord and higher CNS has not been widely studied.
- The extent to which pain is a phenomenon that is distinct from degenerative diabetic neuropathy should be more widely addressed.

Biomarkers

Background

Biomarkers may be considered as being any measurable feature that correlates with some physiological state, or in this case, the pathophysiological progression of diabetic neuropathy. Optimally, identification of the therapeutic efficacy of any drug utilizes three types of biomarkers: (1) target biomarkers identify proteins that directly interact with the target drug; (2) mechanism biomarkers measure some downstream endpoint of drug action and may be behavioral, physiological, genetic, or biochemical; while (3) outcome biomarkers are independent of drug mechanism of action but serve as surrogate markers for clinical efficacy, such as decreasing bacterial count in the case of antibiotics. Advancing the development of novel therapeutics for treating diabetic neuropathy can be greatly facilitated by the identification of selective and sensitive biomarkers indicative of early neurodegenerative changes, prognostic for the continued development of more severe neuropathy, and function as an outcome biomarker for treatment efficacy.

Recommendations

To date, analysis of clinical trial data has identified only one relatively sensitive, but not necessarily selective, biomarker for predicting diabetic neuropathy. Upregulation and phosphorylation of the chaperone protein, Hsp27, is necessary for survival of injured motor neurons and Hsp27 levels increase in sensory ganglia of diabetic rats. In a cross sectional, nested, case–controlled study from the EURODIAB Prospective Complications Study, elevated plasma Hsp27 levels conferred a twofold increased odds ratio of diabetic neuropathy, independent of other conventional risk factors, inflammatory markers, or

albumin excretion rate (Gruden et al., 2008). Indeed, despite a large percentage of the subject cohort manifesting more than one diabetic complication, Hsp27 was found to only segregate with neuropathy, but not retinopathy, macroalbuminuria, or cardiovascular disease. Thus, in the context of diabetic complications, Hsp27 seems to be a selective marker. However, its role in promoting neuronal survival may broaden its selectivity to other neurodegenerative diseases as well. Nonetheless, this protein serves as a valid functional endpoint to assess in animal models of diabetic neuropathy and has clear relevance to the human disease.

At this juncture, disappointingly, there are no other validated biomarkers that can be identified for use in animal models.

Controversies and limitations

While there are no major controversies in the field, identification of biomarkers is a slow and expensive process. Both animal and human samples from multiple biological sources including serum, urine, and cerebrospinal fluid are required. In addition, the usefulness of these samples is dictated by the stage and duration of disease. One category of potential biomarkers, oxidizing intermediates, can be difficult to detect *in vivo* because they are short-lived and generated at low levels. This has required investigators to consider monitoring stable end products of protein and lipid oxidation. Correct analyses to detect these products, present at the sub-femtomole level, combine gas chromatography (GC) with isotope dilution mass spectrometry (MS) and triple quadrapole MS. This approach can establish the molecular fingerprints for oxidative damage *in vivo* in animals and humans with neuropathy. However, they are, as indicated above, time consuming and expensive.

Research opportunities

The absence of current biomarkers for diabetic neuropathy makes this an area of vast opportunity.

- Participants noted that considerably more use must be made of blood and urine samples from animal studies. Nitrosylated proteins in monocytes, Ca^{2+} imaging of blood cells, sorbitol levels in blood, urinary levels of TNFα and isoprostanes could be analyzed in an attempt to identify early signs of disease. The appearance of the biomarkers could then be correlated with progression of diabetic neuropathy.
- The use of non-invasive imaging techniques to assess nerve fiber loss or spinal cord involvement should be encouraged. Transgenic mice with GFP/YFP expression in neurons (under thy1 promoter for example) could be ideal for screening purposes. Imaging of the skin can be performed using standard microscopy or more complex 2-photon imaging.
- Quantification of IENF levels in skin could be simplified by using ELISA to assess PGP9.5 levels. This would entail developing techniques to separate the epidermis, such as blister formation.
- Continue to refine immunofluorescent and/or immunohistochemical analysis of the skin, epidermis, and dermis using specific markers for neurons and Schwann cells.

For example, neuronal markers should include neurofilaments, PGP 9.5, Trk A, TRP channels, casper, and Na^+ channels. For Schwann cells, SC-P0, MBP, MAG, S100b, gliomedin, and Erb B are pertinent. Pan cell markers associated with cell damage such as 4-hydroxy-2-nonenal, nitrotyrosine, PARP, sRAGE, and protein carbonyls could also be optimized.

- The painstaking process of identifying stable oxidative intermediates using mass spectrometry and other emerging techniques should be continued.

General Recommendations

A clear definition of diabetic neuropathy in animal models is needed to promote a better understanding of disease pathogenesis and the development of effective therapies. All workshop attendees agreed that no one set of phenotyping criteria can be developed that is applicable to all rodent models of diabetic neuropathy and that choice of model may vary depending on the question being addressed. Nonetheless, a set of guiding principles for animal phenotyping can be outlined, with recognition that they may not apply to all experimental paradigms. With these caveats in mind, workshop attendees agreed upon the following general principles for phenotyping diabetic rodents.

Experimental design

A number of discussion groups raised concerns about experimental design and analysis that are applicable across all aspects of study:

- Whenever possible, analyses should be conducted with randomized samples and the observer unaware of experimental group identity in order to reduce unintentional bias.
- The number of animals entered into a study and subsequent survival rates should be reported, with the goal of sampling enough animals such that data is both statistically unequivocal and biologically relevant.
- Inclusion of pre-diabetic measurements and age-matched control animals is essential for interpreting whether a diabetes-associated change has a maturational component.
- Establishing the diabetic status of animals should involve careful monitoring and subsequent reporting of body weight, blood glucose (both minimally require onset and terminal values), HbA1c and, depending on the type of diabetes, blood pressure, and plasma insulin and lipids.
- Investigators should always report the husbandry conditions (light : dark cycle, bedding, number of animals per cage) and diet type.
- An a priori plan for appropriate statistical analysis of defined endpoints should be followed with both negative and positive data reported.

Neuropathy phenotyping

- Diabetic neuropathy is defined as the presence of statistically different values between diabetic and age-matched control animals in 2 of 3 assessments (behavior, nerve conduction velocity and/or nerve structure).
- The behavioral test of choice for small fiber neuropathy is the paw withdrawal response latency to escalating heat applied to the plantar surface (Hargreaves method with stimulation of a single paw). The assessment of the paw withdrawal response to light touch of plantar surface (von Frey monofilaments or automated pressure filaments) provides an alternative, albeit potentially more subjective, behavioral assessment of large fiber dysfunction.
- Nerve conduction studies should include the assessment of motor and sensory nerves.
- Nerve structure can be assessed by quantifying IENF density (small fibers) or by nerve trunk morphometry (large and small fibers).
- Serial measurements over the time course of diabetes should be made wherever possible.
- Confirmation of any observation by other independent investigators is essential to establishing the phenotype of a particular animal model.

Summary

The Diabetic Neuropathy Study Group of the EASD reached a consensus on the criteria for establishing the presence of somatic diabetic neuropathy in rodent models. The Study Group recommends that animal phenotyping should consist of at least two of the following:

- Behavior: one or more assessments of nocifensive behavior to establish a measure of pain and/or sensory loss.
- Physiology: nerve conduction studies of motor and sensory nerves.
- Structure: a measure of nerve structure, preferably IENF density if pertinent.

Neuropathy is considered to be present when at least two of three measures are statistically different in diabetic rodents compared to non-diabetic, age-matched, controls. These criteria parallel those used in patients to diagnose diabetic neuropathy and are anticipated to facilitate the development of meaningful animal models of disease.

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