



Ann Arbor VA
Healthcare System

Diabetes-Induced Allodynia: Correlation Between Behavioral Intensity, Duration of Diabetes, and Periaqueductal Gray (PAG) Activation



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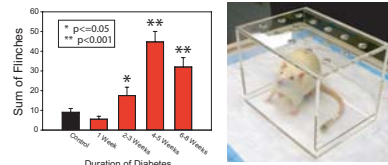
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INTRODUCTION

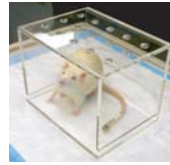
Peripheral polyneuropathy is a common complication of Type 1 diabetes mellitus (DM) and often leads to abnormal pain perception, including mechanical and thermal allodynia or hyperalgesia and severe unremitting spontaneous chronic pain. While, traumatic injury to the peripheral nervous system is known lead to long lasting changes and reorganization within CNS structures involved in pain processing, we know little about the supraspinal mechanisms in painful diabetic neuropathy.

Freshwater and Calcott showed that diabetic rats exhibit a protracted period of finching behavior in response to a 0.2% formalin stimulus during the late phase of the formalin test. Because this stimulus elicits only a few or no finches in non-diabetic rats, this nociceptive behavior in DM rats has been termed low-dose formalin chemogenic allodynia. Previously, we showed that 4-6 week DM rats exhibiting this chemogenic allodynia also showed a significant decrease in the activation of the periaqueductal gray (PAG), a structure involved in antinociception. This finding suggested that reduced PAG activation might be a key CNS mechanism for the development of neuropathic pain in diabetes. Accordingly, we used quantitative behavioral testing combined with neuroimaging, immunohistochemistry and statistical correlation analysis to characterize the inter-relationships between the behavioral intensity of chemogenic allodynia, the duration of diabetes, changes in PAG activation, and blood glucose levels in the streptozotocin (STZ) model of Type 1 diabetes. In addition, to identify the potential cause of the decreased activation in a PAG, we examined whether there was evidence of diabetes-induced apoptotic cell loss in PAG.

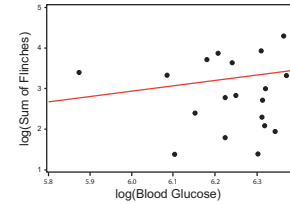
1 STZ-diabetic rats exhibit a marked chemogenic allodynia in response to a low-dose (0.2%) formalin stimulus, which develops over time and lasts for up to 8 weeks.



* Asterisks indicate significant difference from control

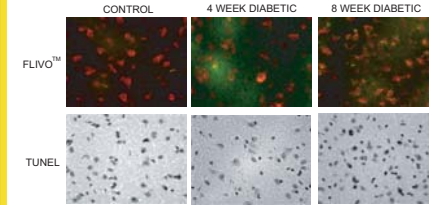


3 Behavioral responses indicative of chemogenic allodynia show no significant correlation to the of blood glucose levels of diabetic rats.



The regression equation for the natural log of the sum of finches versus the natural log of glucose is:
natural log of the sum of finches = $-2.3703 + 0.9054 \cdot \log(\text{glucose})$.
The 95% bootstrapped confidence interval for the slope is (-0.06221, 4.33653).
It contains zero; thus, the slope is not statistically significantly different from zero.

6 As compared to healthy controls, STZ-diabetic rats show evidence of apoptotic cell death in the ventral lateral PAG, indicated by increased caspase activation and an increased number of TUNEL positive neurons.



Representative fluorescent and bright field digital images of the ventral lateral periaqueductal gray from control and diabetic rats (40X field, Nikon 801 microscope equipped with a CoolSnap™ camera).

FLIVO™: Thirty minutes prior to euthanasia, green FAM-FLIVO™, an in-vivo apoptosis detection reagent (Immunochemistry Technologies, LLC) was injected intravenously to directly label dying caspase-positive apoptotic neurons within the live brain. After euthanasia, 10-20μ frozen brain sections through the periaqueductal gray were prepared and counter-stained with red fluorescent Nissl to identify all neurons. Dying apoptotic neurons exhibit dual staining with FAM-FLIVO™ (green) and Nissl (red).

TUNEL™: As a confirmation of apoptosis, separate 10μ brain sections through the periaqueductal gray were prepared for bright-field microscopy using a standard TUNEL stain (Neurotacs II, Trevigen Inc).

METHODS

Subjects: Male Sprague Dawley rats (250-275 gm at start of experiment)

Induction of Diabetes

- Streptozotocin (STZ) injection (45 mg/kg in sterile saline, i.p.)
- Blood Glucose Measurement: pre-STZ baseline, 72 hrs post-STZ, then weekly for up to 8 weeks (diabetes = blood glucose \geq 300 mg/dl)

Formalin-Evoked Allodynia (Chemogenic allodynia)

- 50 μ l s.g. injection of 0.2% formalin in sterile saline into dorsum of left hindfoot
- count paw finches in 1 minute blocks every 5 minutes for 30 - 35 minutes.

Functional Imaging - Brain Activation Studies

- Autoradiographic measurement of regional cerebral blood flow (rCBF) as an index of neuronal activation, using ^{99m}Tc-exametazime (Ceracat™, Amersham Health Sciences).

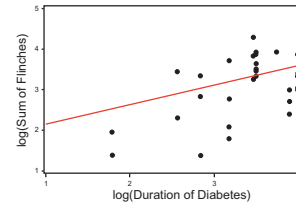
Detection of Apoptosis:

- in-vivo green fluorescent (carboxyfluorescein, FAM) activated polycaspase probe, FAM-FLIVO™ (Immunochemistry Technologies, LLC), to quantitate the level of activated caspases as indicator of apoptosis in the periaqueductal gray (PAG)
- immunohistochemical TUNEL stain (Neurotacs™, Trevigen, Inc.) to confirm apoptotic cell death in PAG

Data Analysis

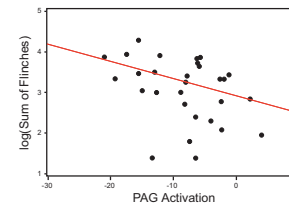
- mixed model ANOVA corrected for repeated measures (SPSS for Windows)
- Regression Analysis (SAS for Windows): The sum of finches was regressed upon the log of glucose, the log of dm duration, and PAG regional activity with a generalized linear model (GLM) using a log link with an appropriate scale adjustment parameter to compensate for the potential presence of overdispersion. To test if the slope of the model was statistically significantly different from zero, 1500 bootstrapped samples were generated and used to construct a 95% confidence interval for the slope of the model. If this confidence interval contained zero, the slope was not considered to be statistically significantly different from zero. If the interval was bounded away from zero, the slope was considered to be statistically significantly different from zero.

2 Behavioral responses indicative of chemogenic allodynia increase progressively as a function of the duration of diabetes.



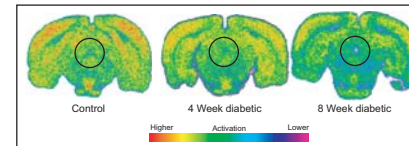
The regression equation of the natural log of sum of finches versus natural log of dm duration is:
natural log of the sum of finches = $1.6568 + 0.4833 \cdot \log(\text{dm duration})$.
The 95% bootstrapped confidence interval for the slope is (0.10962, 0.83382).
It does not contain zero; thus, the slope is statistically significantly different from zero.

4 Periaqueductal Gray (PAG) activation shows a significant negative correlation with behavioral intensity. As PAG activation decreases, there is an increase in the number of finches indicating chemogenic allodynia.

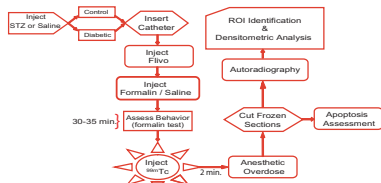


The regression equation for the natural log of sum of finches versus PAG activation is:
natural log of the sum of finches = $2.3160 - 0.0427 \cdot \text{PAG}$.
The 95% bootstrapped confidence interval for the slope is (-0.07111, -0.01205).
It does not contain zero; thus, the slope is statistically significantly different from zero.

5 PAG activation decreases with duration of diabetes, consistent with the negative correlation between PAG activation and behavioral intensity.



Experimental Flow Diagram



SUMMARY & CONCLUSIONS

- (1) The behavioral intensity of low-dose formalin-induced (chemogenic) allodynia is positively correlated with the duration of diabetes, but appears independent of the degree of hyperglycemia in DM rats.
- (2) The negative correlation in diabetic rats between level of behavioral intensity (chemogenic allodynia) and activation in the periaqueductal gray (PAG) supports the hypothesis that neuropathic pain in diabetes is in part the result of reduced activity in supraspinal structures involved in antinociception.
- (3) Increased caspase activity (FAM-FLIVO™ labeling) and high levels of confirmed cell loss (TUNEL positive cells) in the ventral lateral periaqueductal gray are consistent with the idea that diabetes-induced neuronal cell death is a key factor in producing the decreased activation in the PAG described here.