Progressive postnatal decline in leptin sensitivity of arcuate hypothalamic neurons in the Magel2-null mouse model of Prader–Willi syndrome

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Abstract

Prader–Willi syndrome (PWS) is a multigene disorder associated with neonatal failure to thrive, developmental delay and endocrine abnormalities suggestive of hypothalamic dysfunction. Children with PWS typically develop overt hyperphagia and obesity ~8 years of age, later than children with other genetic forms of obesity. This suggests a postnatal developmental or degenerative component to PWS-associated obesity. De novo inactivating mutations in one PWS candidate gene, MAGEL2, have been identified in children with features of PWS. Adult mice lacking Magel2 are insensitive to the anorexic effect of leptin treatment, and their hypothalamic pro-opiomelanocortin (POMC) neurons fail to depolarize in response to leptin. However, it is unclear whether this leptin insensitivity is congenital, or whether normal leptin sensitivity in neonatal Magel2-null mice is lost postnatally. We used in vitro cytosolic calcium imaging to follow the postnatal development of leptin responses in POMC neurons in these mice. Leptin caused an activation of POMC neurons in wild-type acute hypothalamic slice preparations at all ages, reflecting their normal leptin-invoked depolarization. Normal leptin responses were found in Magel2-null mice up to 4 weeks of age, but the proportion of leptin-responsive POMC neurons was reduced in 6-week-old Magel2-null mice. The number of α-melanocyte-stimulating hormone immunoreactive fibers in the paraventricular hypothalamic nucleus was also reduced in mutant mice at 6 weeks of age. A similar progressive loss of leptin sensitivity caused by loss of MAGEL2 in children with PWS could explain the delayed onset of increased appetite and weight gain in this complex disorder.

Introduction

Prader–Willi syndrome (PWS) is a genetic disorder characterized by neonatal hypotonia, intellectual disability, endocrine dysfunction and obesity. Neonatal failure to thrive is followed by a phase that includes weight gain and increased interest in food around the age of 5, before the onset of overt hyperphagia and consequent obesity around the age of 8 (1,2). Although caloric intake is normal for age during childhood, it is excessive given reduced resting energy expenditure (1). Leptin is a hormone that is produced by adipose tissue and acts through leptin receptors on a variety of central nervous system neurons to ultimately regulate energy homeostasis. Children with PWS have excess serum leptin commensurate with their excess fat mass (3), but their behavior paradoxically recapitulates a starvation response. Specifically, their reduced voluntary activity, increased partitioning of available energy into fat, and increased foraging for food (1) are also seen in low leptin or leptin-insensitive conditions. Indeed, children carrying mutations in the genes encoding leptin or its receptor also become severely hyperphagic and obese (4). However, these children typically have increased appetites even as infants, leading to body weights far above the 99th percentile by 1.5 years of age (5,6), in contrast to infants with PWS. We previously proposed that the most parsimonious explanation for obesity and hyperphagia in adults with PWS is a dysfunction in leptin signaling or in responses downstream of the leptin
pathway (7,8). However, the reason for the transition from normal appetite and weight in early childhood in children with PWS to hyperphagia and obesity later in childhood is unknown.

MAGEL2 is one of several genes inactivated in PWS (9–11). A critical role for MAGEL2 in PWS is supported by the identification of six individuals with neonatal hypotonia, developmental delay and autism and varying degrees of hyperphagia and obesity, and who carry de novo protein-truncating mutations in MAGEL2 (12,13). These individuals either met PWS clinical criteria or have a ‘Prader–Willi-Like syndrome’ (OMIM #615547). In mice, Magel2 is most highly expressed in the hypothalamus including the arcuate nucleus (ARC), which is a key region of the brain controlling energy homeostasis (11,14,15). Disruption of Magel2 in mice causes increased adiposity with leptin insensitivity, reduced locomotor activity, reduced fractional lean mass and progressive infertility (7,15–18). Like children with PWS, mice lacking expression of the Magel2 gene (Magel2-null mice) have poor weight gain before weaning, but then develop increased adiposity (19). Magel2 is required in adult mice for normal anorexic responses to leptin. Although adult wild-type (WT) mice normally reduce their food intake following an intraperitoneal injection, this reduction in food intake does not occur in Magel2-null mice (7). Activation of leptin receptors on pro-opiomelanocortin (POMC) neurons in the ARC normally activates downstream pathways, promoting phosphorylation of the transcription factor STAT3, neuronal depolarization and release of the POMC-derived anorexigenic α-melanocyte-stimulating hormone (α-MSH) (20). POMC neurons in brain slices of the ARC of adult Magel2-null mice do not depolarize after leptin application, in contrast to those of WT mice, as measured by electrophysiology in vitro (7).

It is unclear whether this leptin insensitivity is congenital, or whether neonatal Magel2-null mice retain normal leptin sensitivity but become leptin-insensitive postnatally. We reasoned that a postnatally regulated requirement for Magel2 in leptin responses would be more consistent with the delay in onset of increased fat mass in Magel2-null mice compared with mice carrying mutations in leptin or its receptor, which are already obese by 3 weeks of age (21–23). The aim of this study was to examine leptin responses in the hypothalamus of neonatal and juvenile Magel2-null mice. Indeed, we found that leptin responses are normal in POMC hypothalamic neurons of Magel2-null pups until just after weaning, but that the proportion of leptin-responsive neurons then declines as the mice mature into adulthood. A parallel progressive defect in leptin-sensing in the hypothalamus could explain the delayed onset of hyperphagia and weight gain in children with PWS who lack MAGEL2 function. We speculate that, in principle, maintaining the health of critical POMC neurons in early childhood could prevent the onset of hyperphagia and obesity in children with PWS.

Results

Responses to peripherally applied leptin in postnatal day 10 Magel2-null mice

Leptin receptors are expressed on ARC neurons in the first postnatal week in mice (24). Leptin receptor expression increases at postnatal day 10 (P10), coinciding with a surge in leptin secretion (25) and with the development of neuronal projections from ARC neurons to brain regions that mediate appetite and energy expenditure (26). Intraperitoneal administration of leptin increases the number of pSTAT3 immunoreactive (IR) neurons in the ARC starting at P10 (24,27). We injected Magel2-null or WT control littermate P10 pups with either leptin or saline, and then euthanized the mice after 45 min. Consistent with a previous study (24), we found a significant effect of leptin on the number of pSTAT3-IR cells [two-way analysis of variance (ANOVA), P < 0.0001]. Specifically, we detected 2.7-fold more PSTAT3-IR cells in the ARC of WT leptin-injected pups compared with WT saline-injected pups (Fig. 1A, B and D). Similar results were found for P10 Magel2-null pups (Fig. 1D).

We next examined ARC sections from mice that carry a POMC<sup>EGFP</sup> transgene, which facilitates the detection of POMC neurons by direct visualization of green fluorescent protein (GFP) fluorescence or by anti-GFP immunofluorescence performed concurrently with pSTAT3 immunostaining (Fig. 1C). As discussed above, we observed more doubly labeled (POMC<sup>EGFP</sup>-IR and pSTAT3-IR) neurons in leptin-injected pups compared with saline-injected mice, but no difference between genotypes (Fig. 1E). There was no difference between genotypes in the number of POMC<sup>EGFP</sup> neurons in the ARC at P10 (Fig. 1F). We conclude that P10 mice lacking Magel2 have a normal complement of POMC neurons, and that those neurons activate STAT3 as expected in response to intraperitoneally applied leptin.

Calcium imaging of hypothalamic slices

We first attempted to measure leptin actions in ARC POMC<sup>EGFP</sup> neurons of juvenile mice electrophysiologically as we had done previously in adult mice (7). However, the baseline spontaneous activity at this age was too unstable to reliably detect leptin-induced depolarization. We therefore used multiphoton laser-scanning fluorescence microscopy coupled with calcium imaging in live coronal hypothalamic slices to examine leptin responses in POMC neurons in the ARC during postnatal development. Specifically, we made use of the established view that rises in cytosolic calcium in neurons are mostly due to depolarization of neuronal membrane potential sufficient to activate voltage-gated calcium channels (28–30). POMC<sup>EGFP</sup> neurons were identified in ARC slices by their GFP fluorescence at high (4%) laser power and regions of interest (ROIs) outlined around their somata (Fig. 2A). We then reduced the laser power to 1.5% and injected the ARC with Fluo-4-AM to detect calcium fluxes reflecting neuronal activity (28). Neuron-shaped cells with spontaneous fluorescence intensity changes reflecting calcium transients were noted in all slices and at all ages. Although the emission peaks of enhanced green fluorescent protein (EGFP) and Fluo-4 overlap, the EGFP was barely visible at the lower power needed to detect the activity-related Fluo-4 signals (Fig. 2B and C). After a 1 min recording of baseline spontaneous activity, which did not differ between age groups and genotypes, leptin was added to the bath to test for its depolarizing effect (20).

Recordings were obtained for each of the previously identified POMC<sup>EGFP</sup> neurons. Neurons were categorized as responsive if the initial rise in fluorescence intensity occurred at 90–100 s after the start of leptin application (the time needed for the hormone to reach the recording chamber), and the peak fluorescence intensity was at least 10% over baseline (Fig. 2D–F). Only POMC<sup>EGFP</sup> neurons that showed a notable calcium rise upon application of glutamate 10–15 min after recovery from leptin were used for these analyses, to ensure the viability of leptin-unresponsive POMC neurons. In 12-week-old adult WT mice, 31% (12/39) of POMC<sup>EGFP</sup> neurons responded to leptin with a rise in calcium (Fig. 2G), whereas only one of 51 such cells responded to leptin in 6 slices from 3 Magel2-null mice of the same age. This finding
is consistent with our previous electrophysiological study demonstrating a lack of depolarizing leptin responses in POMC neurons in adult Magel2-null mice (7). However, a comparable proportion of leptin-responsive neurons was present in Magel2-null versus WT slices from 3-week-old mice (Magel2-null 28/80 versus WT 13/47). Compared with WT mice, fewer POMC neurons were leptin-responsive in slices from Magel2-null mice at 6 weeks (Magel2-null 7/69 versus WT 40/94, P < 0.0001). In summary, the proportion of POMC neurons that are responsive to leptin is normal until at least 4 weeks of age in Magel2-null mice, but fewer POMC neurons are responsive at 6 weeks, and by 12 weeks only rarely do Magel2-null POMC neurons respond to leptin.

**α-MSH immunoreactivity in the paraventricular nucleus**

POMC neurons project to a variety of targets in the brain, including the melanocortin receptor positive neurons that mediate food intake and that are located in the paraventricular nucleus (PVH) (31,32). Magel2-null mice have a normal number of POMC neurons up to 12 weeks of age, but by 16 weeks ∼39% fewer POMC neurons are present (7). We visualized α-MSH-positive fibers and

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**Figure 1.** Hypothalamic POMC neuron activation in response to intraperitoneal leptin injection in postnatal day 10 mouse pups. (A) pSTAT3 immunoreactivity (red) in cryosections of the ARC from mice euthanized 45 min after saline or leptin (2.5 mg/kg leptin) injection. Nuclei are counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). V3, third ventricle. Scale bar = 100 μm. (B) Diagram of hypothalamic regions seen in A (solid line) and C (dotted line). ARH, arcuate nucleus of the hypothalamus, ME, median eminence, V3, third ventricle, p and v, posterior and ventral regions of the dorsomedial hypothalamus, TU, tuberal nucleus. Image from the Allen Brain Reference Atlas. (C) Immunohistochemical double labeling for pSTAT3 (red) and POMC (green) expression in ARC neurons after leptin treatment. Arrows indicate examples of doubly labeled cells (yellow). Scale bar = 100 μm. (D) Numbers of pSTAT3-IR neurons in the ARC of WT or Magel2-null pups injected with saline (sal) or leptin (lept). Two-way ANOVA, *P* < 0.01 between saline- and leptin-injected. (E) Numbers of pSTAT3 and EGFP doubly positive neurons per section in the ARC after saline (sal) or leptin (lept) treatment. Two-way ANOVA, *P* < 0.01. (F) Number of POMC neurons per section in the ARC. For D, E, and F, error bars indicate the standard error of the mean.
terminals in the PVH in ad lib fed, untreated 12-week-old mice. We readily detected α-MSH-IR fibers in the PVH of WT mice, but α-MSH immunoreactivity was reduced in PVH-containing sections from Magel2-null mice (Fig. 3). Thus not only are adult ARC POMC neurons unresponsive to leptin, they either have fewer projections to the PVH or their projections contain less α-MSH.

**Figure 2.** Multiphoton calcium imaging in acute ARC slices reveals an age-dependent decline in leptin-responsive identified POMC<sup>GFP</sup> neurons in Magel2-null mice. (A) Representative image of native EGFP fluorescence (green) in POMC<sup>GFP</sup> neurons at 4% laser power. Arrows in A–C point to a representative ROI around a POMC<sup>GFP</sup> neuron. (B) Same field of view as A except at 1.5% laser power. The POMC<sup>GFP</sup> signal is barely visible at this laser intensity. (C) Same field of view as in A and B after Fluo-4-AM injection, with Fluo-4 green fluorescence visualized at 1.5% laser power. (D) Increases in Fluo-4 fluorescence intensity, indicating rises in cytosolic calcium, in four leptin- and glutamate-responsive POMC<sup>GFP</sup> neurons. Numbers to the right of the traces correspond to ROIs in slice image in F. (E) Sample recordings of four leptin-unresponsive, glutamate-responsive POMC<sup>GFP</sup> neurons. (F) Image of Fluo-4 loaded ARC slice during glutamate application. Cells in ROIs were identified by their native EGFP fluorescence before Fluo-4-AM injection as in A. (G) Percentage of leptin-responsive neurons in ROIs of POMC<sup>GFP</sup> neurons imaged in ARC slices from 3, 4, 6, and 12-week-old WT (blue bars) and Magel2-null mice (red bars). Differences between genotypes were evident at 6 and 12 weeks by Fisher exact test, **P < 0.0001, *P = 0.0001.
Our previous work identified increased circulating leptin, a failure to reduce food intake in response to peripherally administered leptin, and defects in leptin-mediated depolarization of ARC POMC neurons in adult mice lacking Magel2 (7). A lack of leptin-mediated depolarization is predicted to impair the release of the endogenous melanocortin receptor agonist α-MSH, with downstream effects on appetite and energy expenditure. Here, we demonstrate that mice lacking Magel2 protein retain leptin sensitivity in POMC neurons into the early postweaning period, but that these POMC neurons then become leptin-insensitive between postnatal weeks 4 and 6. Leptin insensitivity in Magel2-null mice is reflected in a reduced calcium rise in POMC neurons after leptin application between 4 and 6 weeks of age (this study), and an impaired anorectic response to intraperitoneal leptin injection at 6 weeks of age (7). Importantly, this is the same time period during which we previously noted the largest rate of weight gain in Magel2-null mice. However, it precedes the period in which the Magel2-null mice are measurably overweight and overfat, at 10–12 weeks. The immediate postweaning period is critical for both the impaired physiology of mice lacking Magel2 and in the transition from failure to thrive to increased weight gain in children with Prader–Willi syndrome.

POMC neurons and neurons expressing the melanocortin receptors are present before birth but the formation of synaptic connections between these two neuronal types occurs postnatally. In mice, ARC POMC neurons are born between E11 and E13, and ∼3000 POMC neurons are present in the adult hypothalamus (20). Peripheral leptin administration evokes responses in POMC neurons that can be measured by STAT3 and ERK phosphorylation even at P10, although exogenous leptin only has a physiological anorectic effect in rodents after P21. The melanocortin receptors in the PVH can be activated from P5–P15, although the synaptic inputs to parvocellular melanocortin receptor-expressing PVH neurons begin to form at P8 and fully develop between P14 and P28 (26,33,34). This timing correlates with the transition to independent ingestive behavior in rodent pups, but occurs earlier, in the third trimester, in humans (35).

Notably, the density of PVH innervation by ARC projections is highly plastic before weaning, and is inhibited in offspring of diet-induced obese rats, in mouse pups nursed by high fat diet-fed dams and in the absence of leptin signaling (36–38).

Here we observe that Magel2-null mice have a normal number of ARC POMC neurons at postnatal day 10, and that these cells respond normally to exogenous leptin by phosphorylation of STAT3. Moreover, calcium imaging of ARC POMC neurons in acute slices demonstrated that a normal proportion of POMC neurons is activated by leptin at P10. Although the POMC neurons are activated by leptin at P10. Although it is not possible to measure leptin-mediated activation at birth because of low leptin receptor expression in the ARC (24), it is likely that Magel2-null mice are born with a normal number of eventually leptin-sensing ARC POMC neurons. A normal proportion of leptin-activated POMC neurons were seen in 3 and 4-week-old Magel2-null mice, but fewer neurons responded to leptin by 6 weeks of age and virtually no responders were seen in 12-week-old mutants. Strikingly, the age at which Magel2-null mice first
display a reduction in the proportion of leptin-responsive POMC neurons coincides with the period of fastest weight gain (17). This suggests that protection of leptin-sensing POMC neurons from degeneration could reduce excessive fat gain and increase voluntary activity in mice lacking Magel2.

Last, we found a reduced density of α-MSH axonal projections from the ARC to the PVH in Magel2-null mice. This condition mimics that of rodents that lack leptin receptor signaling completely or lack the ability to activate pSTAT3 in POMC neurons, which likewise have a reduced density of projections from POMC neurons onto PVH neurons (24). Other conditions that affect the ability of POMC neurons to innervate the PVH include dysfunction in autophagy, which inhibits POMC neurite outgrowth and reduces the density of α-MSH projections to the PVH (39).

Our study was motivated by the contrast between Magel2-null mice, which gain weight only after weaning, and animals that lack leptin signaling entirely (Leprdb and Lepr null mice and Zucker fa/FA rats), which are already obese by 3 weeks of age (21–23). Interestingly, loss of leptin receptor signaling just in POMC neurons produces a remarkable phenotype of increased fat mass without a measurable change in energy expenditure or food intake, with excess weight starting between 4 and 6 weeks of age (40). Inactivating the signaling protein STAT3 just in POMC neurons likewise causes mild obesity and defects in compensatory refeeding similar to those we identified in Magel2-null mice (41). Inactivating ciliary function just in POMC neurons is sufficient to cause obesity, while impairing autophagy just in POMC neurons causes an adult-onset increase in body fat and only a small increase in body weight, also similar to the Magel2-null phenotype (39,42). ARC POMC neurons appear to be highly vulnerable to dysfunction in a number of situations, including deficient ubiquilatation (43), high fat feeding, microRNA dysregulation (44) and endoplasmic reticulum stress (45,46). MAGEL2 is important for endosomal protein recycling (47), a process implicated in neurodevelopmental and neurodegenerative disorders (48–50). Further experiments are required to determine whether POMC neuron dysfunction is caused by the loss of Magel2 in the POMC neurons themselves, or loss of Magel2 in other cells that are required for the health of POMC neurons. Congenital absence of MAGEL2 could cause defective axonal outgrowth from ARC POMC neurons and deterioration in their leptin responsiveness. Future studies that examine the leptin–melanocortin function in individuals with PWS and MAGEL2 deficiency could facilitate the development of treatments targeted at protecting POMC neurons from functional changes in leptin-mediated responses, potentially delaying or preventing hyperphagia in children with PWS.

Materials and Methods
Animals and husbandry

The University of Alberta Animal Care and Use Committee approved all procedures involving animals in accordance with the guidelines of the Canadian Council on Animal Care. Magel2-null mice and WT control littermates (C57BL/6-Magel2tm1Stw/J, The Jackson Laboratory stock #00962) were generated as described (16,17). In some cases, Magel2–/–Pp carrier males were crossed with homozygous POMC<sup>GFP</sup> reporter mice, expressing GFP in POMC+ cells (stock #099593) (20). This cross produced Magel2–/–Pp; POMC<sup>GFP</sup> experimental offspring that lack expression of Magel2 but express POMC<sup>GFP</sup>. Magel2<sup>+/–</sup>P, POMC<sup>GFP</sup> littermates expressing Magel2 and the POMC<sup>GFP</sup> reporter gene were used as controls. Mice used as adults were weaned between 3 and 4 weeks of age and then group housed under a 12:12 light:dark cycle with food (PicoLab Rodent Diet 5001) and water available ad lib. We found no sex-specific differences in any of the measurements reported here so have combined data from both sexes for the analysis.

Leptin stimulation and immunofluorescence microscopy

Non-fasted pups were injected intraperitoneally with either 2.5 mg/kg recombinant mouse leptin (National Hormone and Peptide Program, NHPP-NIDDK, Torrance, CA), or phosphate-buffered saline (PBS) (pH 7.4) 45 min before euthanasia, then perfused intracardially with 2% paraformaldehyde in PBS. A block containing the hypothalamus was cut free from the dissected brain, postfixed and cryoprotected in PBS with 20% sucrose. Cryo-sections (30 µm) were collected rostral to the ARC (for PVH) or through the entire ARC, as verified by thionine staining of adjacent sections. For pSTAT3 and GFP double immunofluorescence staining, sections were chosen to maximize the number of POMC neurons (7,20). pSTAT3 was detected with a rabbit anti-pSTAT3 antibody (1:500, Cell Signaling 9131) and a goat-anti-rabbit secondary antibody (1:500, AF564, Life Technologies). For double labeling, sections were washed, pretreated, then incubated with a chicken anti-GFP antibody (1:1000, Abcam 13970) followed by a goat-anti-chicken secondary antibody (1:1000, Life Technologies AF488). To detect α-MSH-IR fibers or terminals in the PVH, sections were incubated with a rabbit anti-α-MSH antibody (1:4000 Abcam 123811) followed by a goat-anti-rabbit secondary antibody (1:500, Life Technologies AF564). Sections were washed in PBS, counterstained with 4’,6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei and mounted with ProLong Gold Antifade reagent (Life Technologies).

Cell imaging and counting

Sections were imaged using the Zeiss LSM 510 confocal microscope and Leica Application Suite Advanced Fluorescence (LAS AF) software. For pSTAT3-IR and EGFP-IR, cells were counted manually using Image J (NIH). As previously described, α-MSH-IR fibers in the PVH were imaged, binarized to optimize detection of IR fibers, then pixels were counted to estimate the density of IR fibers in each region (26). Differences in the number of IR cells or IR fibers between genotypes and between treatment conditions were analyzed by two-way ANOVA (GraphPad Prism version 5). P < 0.05 was considered statistically significant. We analyzed at least three mice per group and three sections per mouse.

Slice preparation and calcium imaging

Brains from 3 to 12-week-old POMC<sup>GFP</sup> reporter mice were prepared and sliced (250 µm) in carboxenated ice-cold artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 3 KCl, 1.3 MgSO<sub>4</sub>, 1.4 NaH<sub>2</sub>PO<sub>4</sub>, 2.5 glucose, 7.5 sucrose, 26 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub>, 1 kynurenate, pH 7.4 (300–305 mOsm) then placed in carboxenated room-temperature aCSF without kynurenate (7). Recordings were performed with a ‘multiphoton exclusive’ microscope (Olympus, Richmond, ON, Canada) equipped with a 20× XLUM PlanFI-NA-0.95 objective. A Z-stack was first obtained at ×600 magnification through a portion of the ARC slice using multiphoton microscopy to visualize POMC neurons. The laser power was set to 4% to permit detection of the uniformly weak native EGFP fluorescence. The laser power was then reduced to 1.5% and the slice injected with 3 µl Fluo-4-AM dye (0.5 mM in aCSF) using a thin-walled glass patch pipette broken to a tip diameter of 5–15 µm (28). For cytosolic calcium imaging, a focal plane was chosen that contained the largest number of visualized POMC<sup>GFP</sup>
neurons. To ensure that the final image acquisition contained this confocal plane, adjacent planes 5 μm apart and bracketing the chosen plane were also scanned at 1.1 s intervals throughout the recording. XYT scans resulting in time-lapse videos were recorded continuously for 20 min. Leptin (100 nM, 10 ml) was bath-applied for 30 s to verify neuronal viability and allowed to wash out for 10–15 min after the initiation of each recording for 1 min, then allowed to wash out until the end of the recording. Image files were analyzed using FluoView v4.3 software. POMC EGFP neurons were identified in a calcium recording by matching slice landmarks in a still frame capturing the peak glutamate response with a plane from the Z-stack collected prior to Fluo-4-AM injection. These images were used to place ROIs over POMC EGFP neurons. Any change in fluorescence intensity in excess of 10% of baseline was considered statistically significant.

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