The histopathological hallmarks of Alzheimer disease (AD) include intraneuronal neurofibrillary tangles composed of abnormally hyperphosphorylated \( \tau \) protein. Insulin dysfunction might influence AD pathology, as population-based and cohort studies have detected higher AD incidence rates in diabetic patients. But how diabetes affects \( \tau \) pathology is not fully understood. In this study, we investigated the impact of insulin dysfunction on \( \tau \) phosphorylation in a genetic model of spontaneous type 1 diabetes: the nonobese diabetic (NOD) mouse. Brains of young and adult female NOD mice were examined, but young NOD mice did not display \( \tau \) hyperphosphorylation. \( \tau \) phosphorylation at \( \tau-1 \) and pS422 epitopes was slightly increased in nondiabetic adult NOD mice. At the onset of diabetes, \( \tau \) was hyperphosphorylated at the \( \tau-1, AT8, CP13, pS262, \) and pS422 epitopes. A subpopulation of diabetic NOD mice became hypothermic, and \( \tau \) hyperphosphorylation further extended to paired helical filament-1 and TG3 epitopes. Furthermore, elevated \( \tau \) phosphorylation correlated with an inhibition of protein phosphatase 2A (PP2A) activity. Our data indicate that insulin dysfunction in NOD mice leads to AD-like \( \tau \) hyperphosphorylation in the brain, with molecular mechanisms likely involving a deregulation of PP2A. This model may be a useful tool to address further mechanistic association between insulin dysfunction and AD pathology. *Diabetes* 62:609–617, 2013

**RESEARCH DESIGN AND METHODS**

**Animals.** Female NOD/ShiLtJ (The Jackson Laboratory, Bar Harbor, ME) were used as the onset of type 1 diabetes symptoms occurs earlier and with a higher incidence (60–80%) in comparison with males (20–30%) (17). As these mice were originally derived from outbred Institute of Cancer Research mice (ICR, also available as CD-1 mice) (18), we used ICR (Crl:CD1 [ICR]; Charles River Laboratories, Wilmington, MA) female animals as controls. Animals were handled according to procedures approved by the Comité de Protection des Animaux under the guidelines of the Canadian Council on Animal Care.

**Monitoring of physiological parameters.** The diagnosis of diabetes was done by monitoring the mice for glycosuria, glyceremia, and insulinemia. Mice were considered diabetic when nonfasting plasma glucose level was >12 mmol/L, and there was presence of glycosuria for 2 consecutive weeks (18). Glyceremia was monitored with reagents strips for urinalysis (Diastix; Bayer HealthCare, Pittsburgh, PA). Nonfasting blood glucose was measured using

**Deregulation of Protein Phosphatase 2A and Hyperphosphorylation of \( \tau \) Protein Following Onset of Diabetes in NOD Mice**

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a glucometer with reagent strips (ACCU-CHEK Aviva Nano; Roche Diagnostics, Mannheim, Germany) or a Glucose Assay Kit (Biovision Inc., Mountain View, CA). Plasma insulin was determined using a sandwich enzyme immunoassay according to the manufacturer’s instructions (Mouse Insulin ELISA, Merckodia, Sweden). All mice were weighed at sacrifice, and the body temperature was monitored using a rectal probe (Thermalert TH-5; Physitemp, Clifton, NJ).

Protein extraction. Mice were killed by decapitation without anesthesia because anaesthesia can increase hypothermia-induced \( \tau \) phosphorylation (19). Brains were immediately removed, and tissues were dissected on ice. Cortical tissues were quickly weighed, frozen on dry ice, and maintained at \(-80^\circ C\). Protein extraction from frozen samples was performed as described previously (20).

Western blot analysis. SDS-PAGE and Western blot analysis was done as previously described (20). All antibodies used in this study are described in Table 1. Immunoreactive bands were visualized using the ImageQuant LAS 4000 imaging system (GE Healthcare Biosciences, Piscataway, NJ), and densitometric analysis was performed with Image Gauge analysis software (Fujiﬁlm USA, Valhalla, NY).

Immunofluorescence. Tissue fixation was done according to the cold Bouin’s method previously developed in our laboratory (21). Bound antibodies were visualized with Alexa Fluor 568–conjugated anti-mouse IgG (1:500) or Alexa Fluor 488–conjugated anti-rabbit IgG (1:1,000) (Molecular Probes, Eugene, OR). Immunoreactive bands were observed under a Carl Zeiss Axio Imager M2 (Carl Zeiss, Jena, Germany) microscope equipped with a Nuance FX multispectral imaging system (Cambridge Research & Instrumentation, Woburn, MA) and Nuance 2.10 software (Cambridge Research & Instrumentation).

PP2A immunoprecipitation assay. Brain phosphatase activity was determined using the Ser/Thr phosphatase Assay Kit from Millipore (Temecula, CA) according to the manufacturer’s instructions. After immunoprecipitation, the activity for PP2A was assessed by the release of phosphate from a chemically synthesized phosphopeptide over a period of 10 min at 30°C. The amount of phosphate released was measured by the absorbance of the malachite green–phosphate at 630 nm.

Statistical analysis. Statistical analyses were performed with GraphPad Prism software 4.0 (GraphPad). Differences between groups were calculated using a one-way ANOVA followed by a Newman-Keuls post hoc test. Effects were considered significant at \( P < 0.05 \). Protein phosphorylation between 4- and 30-week-old ICR mice was investigated.

RESULTS

Physiological parameters of NOD and ICR mice. We separated our animals into two age groups: young (4 weeks old, \( n = 5 \)) and adult (18–30 weeks old, \( n = 18 \)) mice (Table 2). We followed the development of type 1 diabetes symptoms by measuring several metabolic and physiological parameters including weight, glycemia, insulinemia, glycosuria, and body temperature (Table 2), because we have previously demonstrated that alterations of glucose metabolism can induce hypothermia leading to \( \tau \) hyperphosphorylation (21). At 4 weeks of age, young NOD mice were not diabetic and had lower glycemia in comparison with ICR controls, an observation that is consistent with the literature (17). No significant changes in body temperature were detected in these mice.

Adult NOD mice developed type 1 diabetes symptoms progressively and were accordingly classified into three subgroups (see Table 2 for groups and values). The first subgroup consisted of nondiabetic NOD mice (\( n = 7 \)) with a mean age of 30 weeks. This group did not show any significant changes in blood glucose or insulin levels when compared with their ICR controls. The second group consisted of glycosuric NOD mice (NODG, \( n = 7 \)) with a mean age of 25 weeks. These mice started to develop type 1 diabetes and were characterized by higher glycemia as well as glycosuria. Notably, blood insulin levels of this group of mice revealed hypoinsulinemia in comparison with ICR controls. Interestingly, four diabetic mice showed, in addition to hyperglycemia and glycosuria, a significant drop of body temperature, massive hyperglycemia, as well as hypoinsulinemia compared with ICR controls.
### Antibodies used in this study

| Name | Abbreviation | Epitope | Type | Origin | Provider | WB | IHC |
|------|--------------|---------|------|--------|----------|----|-----|
| **τ** | Anti-τ-1, clone PC1C6 | τ-1 | Non-phospho-S195, 198, 199, 202 | Mono | Mouse | Millipore | 1/1,000 | NU |
| | Anti-PHF-τ, clone AT8 | AT8 | pS202, pT205 | Mono | Mouse | Thermo Scientific | 1/1,000 | NU |
| | Anti-PHF-τ, clone AT100 | AT100 | pT212, pS214 | Mono | Mouse | Thermo Scientific | 1/1,000 | 1/500 |
| | CP13 | CP13 | pS202 | Mono | Mouse | Peter Davies | 1/1,000 | NU |
| | TG-3 | TG-3 | pT231 | Mono | Mouse | Peter Davies | 1/1,000 | NU |
| | Anti-τ (pS262) | PS262 | pS262 | Poly | Rabbit | Invitrogen | 1/1,000 | NU |
| | PHF-1 | PHF-1 | pS396, pS404 | Mono | Mouse | Peter Davies | 1/1,000 | NU |
| | Anti-τ (pS422) | PS422 | pS422 | Poly | Rabbit | Invitrogen | 1/1,000 | NU |
| | Anti-human τ A0024 | Total τ | Human τ COOH terminus | Poly | Rabbit | Dako | 1/5,000 | 1/1,000 |

**Kinases**

| Name | Abbreviation | Epitope | Type | Origin | Provider | WB | IHC |
|------|--------------|---------|------|--------|----------|----|-----|
| GSK-3β | Phospho GSK3β (Ser9) | GSK-3β 1-160 | Poly | Rabbit | BD Transduction | 1/1,000 | NU |
| Akt | Phospho-Akt (Ser473) | pS473 | Poly | Rabbit | Cell Signaling Technology | 1/1,000 | NU |
| p44/p42 MAPK | Phospho-p44/42 MAPK (Erk1/2) | pT202, pY204 | Poly | Rabbit | Cell Signaling Technology | 1/1,000 | NU |
| SAPK/JNK | JNK | Human JNK2 | Poly | Rabbit | Cell Signaling Technology | 1/1,000 | NU |
| Cdk5 (C-8) | P35 | Human p35 COOH terminus | Poly | Rabbit | Santa Cruz Biotechnology | 1/1,000 | NU |
| CamKIIα (A-1) | CaMKII | Mouse CamKIIα 303-478 | Mono | Mouse | Santa Cruz Biotechnology | 1/1,000 | NU |
| Phosphatases | PP1 (E-9) | PP1 | Human full-length PP1-α | Mono | Mouse | Santa Cruz Biotechnology | 1/1,000 | NU |
| Pan-calcineurin A | PP2A A subunit | Human PP2A A subunit (α and β) | Poly | Rabbit | Cell Signaling Technology | 1/1,000 | NU |
| Anti-PP2A, C subunit, clone 7A6 | Anti-PP2A, C subunit | Human PP2A C subunit 302-309 | Mono | Mouse | Millipore | 1/1,000 | NU |
| Demethylated-PP2A-C (4B7) | Demethylated-PP2A-C | Unmethylated PP2A C COOH terminus | Mono | Mouse | Santa Cruz | 1/1,000 | NU |
| | PPP2R2A (2G9) | Rat PPP2R2A/PP2A, B55-α/PR55-α | Mono | Mouse | Cell Signaling Technology | 1/1,000 | NU |
| | PPP2R2B | Human PPP2R2B | Poly | Rabbit | Bethyl Laboratories | 1/1,000 | NU |
| | PP5 | Human PP5 NH₂ terminus | Poly | Rabbit | Cell Signaling Technology | 1/1,000 | NU |

CTF, COOH-terminal fragments; IHC, dilution used in immunohistochemistry; MAPK, mitogen-activated protein kinase; Mono, monoclonal; NU, not used; PKB, protein kinase B; Poly, polyclonal; SAPK, stress-activated protein kinase; WB, dilution used in Western blotting.
We explored the activation patterns of all of these kinases with a panel of antibodies (Table 1).

The nondiabetic adult NOD mice did not show significant changes for any of the investigated kinases as compared with ICR mice (Fig. 3).

By contrast, the onset of diabetes was accompanied by several changes in the expression and activation pattern of τ kinases. Thus, both NODG and NODH mice revealed a significant increase in the phosphorylation state of GSK-3β at the Ser9 epitope, which reflects GSK-3β inhibition (Fig. 3B). Interestingly, we observed a strong increase in AKT phosphorylation in both diabetic NODG and NODH mice, which was accompanied by a significant decrease in the total levels of AKT (Fig. 3C and D), suggesting an activation of this kinase. However, there was no increase in τ phosphorylation in the AT100 epitope (T212/S214), a known in vitro phosphorylation site for AKT (24). As shown in Fig. 4A, levels of PP1 did not significantly change in all groups tested. By contrast, PP2B showed a significant decrease in both nondiabetic NOD and glycosuric NODG mice (Fig. 4B). In addition, we observed a slight increase in the expression of PP5 in NODH mice (Fig. 4H).

PP2A is a heterotrimeric holoenzyme consisting of a core dimer composed of a catalytic subunit (C) tightly bound with the scaffolding (A) subunit, and this core dimer associates with a variable regulatory B subunit. Only trimeric forms of PP2A containing the Bo or Bβ subunits associate with neural microtubules (27). Moreover, PP2A activity is enhanced by the methylation of its catalytic subunit (PP2A C) and is conversely decreased by its demethylation (28).

As our results failed to adequately explain the extent of τ hyperphosphorylation specific to type 1 diabetes in terms of kinase activation, we next assessed the expression levels of τ Ser/Thr protein phosphatases (PP). PP are classified into five types: PP1, PP2A, PP2B, PP2C, and PP5, all of them being highly expressed in the mammalian brain (25). Biochemical studies have previously demonstrated that all PP except PP2C can dephosphorylate τ in vitro (26). We thus examined the profiles of these four PP using specific antibodies (Table 1).

As shown in Fig. 4A, levels of PP1 did not significantly change in all groups tested. By contrast, PP2B showed a significant decrease in both nondiabetic NOD and glycosuric NODG mice (Fig. 4B). In addition, we observed a slight increase in the expression of PP5 in NODH mice (Fig. 4H).

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Regional analysis of \( \tau \) hyperphosphorylation. In AD, the earliest detectable \( \tau \) hyperphosphorylation is localized in neurites of vulnerable neurons before undergoing somatodendritic relocalization (29) and aggregation as NFTs (30). To investigate a possible relocalization of somatodendritic relocalization (29) and aggregation as neurites of vulnerable neurons before undergoing the earliest detectable hyperphosphorylation, we examined the gross anatomical pattern of \( \tau \) phosphorylation by immunofluorescence. Staining of hippocampal sagittal sections clearly showed a robust increase in phospho-\( \tau \) immunoreactivity at the AT8 epitope in NODH mice and to a much lesser extent in the NODG group in comparison with ICR controls (Fig. 5A–C). However, we did not detect significant changes in total or phospho-\( \tau \) cellular localization, as revealed by AT8, total \( \tau \), and DAPI staining patterns (Fig. 5D–I).

Amyloid precursor protein metabolism in NOD mice. We also wanted to address the possibility that changes in amyloid precursor protein (APP) metabolism could be a plausible driving force for altered \( \tau \) phosphorylation in NOD mice (31). Except for a mild decrease in APP full-length in nondiabetic NOD mice, no other significant changes were observed in either APP full-length or APP COOH-terminal fragment levels in adult NOD mice (data not shown). These results confirm our previous data demonstrating that APP metabolism is not affected in mice with STZ-induced type 1 diabetes (10) and suggest that \( \tau \) hyperphosphorylation in NOD mice is not the result of alterations in APP metabolism.

DISCUSSION
In this study, we investigated \( \tau \) phosphorylation and its molecular mechanisms in the NOD mouse strain, one of the most valuable genetic animal models for type 1 diabetes (32). Our data suggest that spontaneous type 1 diabetes provokes a progressive \( \tau \) hyperphosphorylation that begins to be detectable in adult mice even during the nondiabetic stage, in which there is no apparent deregulation of glucose metabolism. We further show that \( \tau \) phosphorylation is greatly exacerbated in the presence of principal type 1 diabetes features, notably hyperglycemia and glycosuria, and further amplified by hypothermia. Finally, we demonstrate that \( \tau \) hyperphosphorylation during type 1 diabetes is likely attributable to a deregulation in PP2A, the major \( \tau \) phosphatase in vivo.

Our findings are consistent with our previous study (10) and several other studies showing \( \tau \) hyperphosphorylation at several epitopes in type 1 diabetes induced by STZ in mice (11–13), or rats (14). However, many of these articles did not document the temperature of the animals. This is important because we have reported before (10), and confirmed in this study, that \( \tau \) phosphorylation is increased when diabetic mice become hypothermic. Indeed, decreased temperature is a common outcome in both human (33) and experimental diabetes (10,34), and hypothermia is a powerful regulator of \( \tau \) phosphorylation, increasing it by 80% per degree Celsius below 37°C in mice (21).

In comparison with our previous study in STZ-treated mice (10), several differences can be noted in the pattern of \( \tau \) phosphorylation with the NOD mice. For example, in this study we observed that the AT8, pS262, and pS422 epitopes were hyperphosphorylated in nonhypothermic NODG mice, whereas these epitopes did not show any significant changes in the absence of hypothermia in STZ-induced animals. In contrast, \( \tau \) phosphorylation at the PHF-1 epitope was increased only in the group of hypothermic NODH mice, whereas we have detected a significant increase in this epitope in nonhypothermic mice.

**FIG. 2.** Effect of type 1 diabetes on \( \tau \) phosphorylation in adult NOD mice. Proteins from mice 18 to 30 weeks of age were extracted from mice cortices, separated by SDS-PAGE, and identified with the following antibodies: A: Tau-1, B: AT8, C: CP13, D: TG3, E: PS262, F: PHF-1, G: PS422, and H: total \( \tau \). Lanes are identified as follows: lanes 1 & 2, ICR control mice; lanes 3 & 4, nondiabetic NOD mice; lanes 5 & 6, diabetic glycosuric NODG mice, and lanes 7 & 8, glycosuric and hypothermic NODH mice. For each epitope, two representative lanes from each group are displayed. Dividing lines represent areas where lanes were removed and the remaining lanes were spliced together. Data are means ± SD. Asterisks indicate significant differences from controls, with *\( P < 0.05 \), **\( P < 0.01 \), and ***\( P < 0.001 \).
following STZ treatment (10). These differences could be due to the fact that NOD and STZ-induced mice develop insulin deficiency that characterizes type 1 diabetes in a differential manner. Indeed, whereas NOD mice showed lower (but detectable) blood insulin levels in comparison with control, insulin levels were not detectable in STZ-induced animals (10). Another possible explanation is that STZ might have a direct impact on tau phosphorylation in the brain. Although peripheral administration of STZ is not thought to impact the brain directly because its transport and cytotoxicity are dependent on the GLUT-2 glucose transporter (35), GLUT-2 has also been found in the mammalian brain, and intracerebroventricular application of STZ in minute amounts has been reported to directly induce hyperphosphorylation (36). Thus, NOD mice might be models more relevant to humans to study the impact of insulin dysfunction on tau phosphorylation during diabetes.

We observed tau hyperphosphorylation at the pS422 epitope in nondiabetic NOD mice prior to deregulation in the glucose metabolism. This epitope is associated with early pretangle formation (37) and the promotion of tau aggregation (38) and is characteristic of abnormal AD-like tau phosphorylation. Concurrent with hyperglycemia and glycosuria in adult NOD mice, tau phosphorylation increased at additional epitopes, notably AT8, CP13, pS262, PHF-1, and pS422. Some of these sites have been linked to specific aspects of tau pathology such as the inhibition of tau microtubules binding (e.g., Ser262) (39) and the promotion of tau aggregation (e.g., Ser396 and Ser422) (38). Moreover, PHF-1 is associated with late-paired helical filament and NFT formation (40). Therefore, it appears that insulin dysfunction results in an increase in tau phosphorylation at epitopes that are critical for the development of tau pathology.

During the nondiabetic stage, adult NOD mice did not show any changes in expression and activation patterns of all investigated kinases. We observed an increase in inhibitory phosphorylation of GSK-3β in both NODG and NODH mice, in accordance with previous studies revealing inhibition of GSK-3β in STZ-induced animal models (10,11,41). However, it should be mentioned that the elevation of GSK-3β Ser9 in NODH mice is not surprising, because GSK-3β Ser9 phosphorylation is a constant result of hypothermia in the mouse brain (21). The increased
phosphorylation of Ser9 could be explained by the activation of AKT observed in NODG and NODH mice. In fact, AKT was the only kinase activated in NOD mice, and its activation failed to explain the extent of hyperphosphorylation at multiple epitopes.

The analysis of different phosphatases in type 1 diabetes animal models is less documented compared with kinases. However, we (10) and others (11,14) have reported that PP2A is inhibited in STZ-induced animal models. In this study, we detected a decrease in the Bα regulatory subunit and an increase in demethylated (inhibition) and total PP2A catalytic subunit in NODG and NODH mice. These changes were paralleled by a significant decrease in the activity of PP2A. Although seemingly counterintuitive, the increase of the catalytic subunit along with a decrease in activity can be explained by the potent autoregulatory mechanism that adjusts PP2A C levels according to PP2A activity, in which inhibition of PP2A leads to the accumulation of the C subunit, either in vitro or in vivo (20,42). These results corroborate our previous observation of increased PP2A C concomitant with decreased PP2A activity in STZ-treated mice (10).

We also observed changes in PP2B and PP5 levels, but among all phosphatases that dephosphorylate τ, PP2A is the major τ phosphatase in vivo, with PP2A, PP1, PP5, and PP2B contributing to 71, 11, 10, and 7%, respectively, of the total τ phosphatase activity in the brain (43).

Importantly, PP2A can regulate the phosphorylation of all the sites studied in this paper (43), and its deregulation is thought to be an important factor in the evolution of AD pathology (26). Taken together, our results suggest that the progressive deregulation of PP2A in NOD mice is likely to be the cause of the observed τ hyperphosphorylation.

An important question is whether it is peripheral and/or central insulin dysfunction that causes PP2A deregulation and τ hyperphosphorylation. Some studies may hint that central insulin dysfunction is not involved in the phenotype of the NOD mice. For example, central insulin dysfunction, whether it is in patients (44) or mediated by knocking out the brain insulin receptor (9) or by direct injections of STZ in the rodent brain (36,45), does correlate with increased τ phosphorylation, but also with decreased AKT and GSK-3β Ser9 phosphorylation, the reverse of what we observed in NOD mice. In contrast, mice treated with STZ peripherally mirror what we observed in NOD mice, namely inhibition of PP2A and augmented phosphorylation of τ, AKT, and GSK-3β (10,11,46). It is interesting to note that the rise in AKT and GSK-3β Ser9

![Image](diabetes.diabetesjournals.org)

**FIG. 4.** Effect of type 1 diabetes on phosphatases in adult NOD mice. Proteins from mice 18 to 30 weeks of age were extracted from mice cortices, separated by SDS-PAGE, and identified with the following antibodies: A: PP1, B: PP2B, C: PP2A A, D: PP2A C, E: Demethylated PP2A C (PP2A C Dem), F: PP2A Bα, G: PP2A Bβ, H: PP5, and I: PP2A activity. Lanes are identified as follows: lanes 1 & 2, ICR control mice; lanes 3 & 4, nondiabetic NOD mice; lanes 5 & 6, diabetic glycosuric NODG mice, and lanes 7 & 8, glycosuric and hypothermic NODH mice. For each epitope, two representative lanes from each group are displayed. Dividing lines represent areas where lanes were removed and the remaining lanes were spliced together. Data are means ± SD. Asterisks indicate significant differences from controls, with *P < 0.05, **P < 0.01, and ***P < 0.001.
phosphorylation in both NOD and STZ-treated mice is probably one of the most valuable and relevant models to study the effects of insulin dysfunction on \(\tau\) phosphorylation in the brain. Crossing NOD mice with mouse models that express human \(\tau\) and develop NFTs might further help us to understand the impact of diabetes on the pathogenesis of AD.

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M.-A.P. and N.B.E.K. performed the experiments and wrote the manuscript. F.Ma., C.J., F.Mo., A.B., and F.R.P. performed the experiments. S.G., A.A., and P.M.M. contributed to the research design and methods and the discussion sections and reviewed the manuscript. S.S.H. contributed to the discussion and reviewed the manuscript. E.P. designed the experiments and wrote the manuscript. E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIG. 5. Regional anatomical localization of phosphorylated \(\tau\) protein in adult NOD mice. Unmixed fluorescent photomicrographs of hippocampal sagittal sections are shown with AT8 (Red, A–C), Total Tau (Green, D–F), or merged with DAPI (G–I), for the following conditions: control (ICR mice, A,D,G), glycosuric NODG mice (B,E,H), or glycosuric and hypothermic NODH mice (C,F,I). All images were taken at original magnification ×5.
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