Selective T-Type Calcium Channel Blockade Alleviates Hyperalgesia in *ob/ob* Mice

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OBJECTIVE—Morbid obesity may be accompanied by diabetes and painful diabetic neuropathy, a poorly understood condition that is manifested by mechanical or thermal allodynia and hyperalgesia. Recent studies have highlighted the importance of T-type calcium channels (T-channels) in peripheral nociception; therefore, our goal was to examine the function of these channels in the pathophysiology and development of painful diabetic neuropathy.

RESEARCH DESIGN AND METHODS—In vivo testing of mechanical and thermal sensation, morphometric peripheral nerve studies, and electrophysiological and biochemical measurements were used to characterize the role of T-channels and the development of painful diabetic neuropathy in leptin-deficient (*ob/ob*) mice.

RESULTS—We found that *ob/ob* mice developed significant mechanical and thermal hypersensitivity early in life that coincided with hyperglycemia and was readily reversed with insulin therapy. These disturbances were accompanied by significant biophysical and biochemical modulation of T-channels in dorsal root ganglion neurons as measured by a large increase in the amplitude of T-currents and the expression of mRNA. The most prevalent subtype, α1H ($Ca_v3.2$), was most strongly affected. Moreover, (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile (ECN), a novel neuroactive steroid and selective T-channel antagonist, provided dose-dependent alleviation of neuropathic thermal and mechanical hypersensitivity in diabetic *ob/ob* mice.

CONCLUSIONS—Our results indicate that pharmacological antagonism of T-channels is potentially an important novel therapeutic approach for the management of painful diabetic neuropathy. ***Diabetes*** **58:2656–2665, 2009**

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It is predicted that more than 200 million people worldwide will have type 2 diabetes by 2025 (1). A common complication of diabetes is peripheral diabetic neuropathy (PDN), which is often marked by mechanical and thermal allodynia and hyperalgesia and cannot yet be treated effectively because of the lack of knowledge of its pathophysiology (2,3).

Recent studies with leptin-deficient (*ob/ob*) mice, an animal model of morbid obesity and type 2 diabetes (4), have suggested that PDN is commonly manifested as mechanical allodynia (5), nerve conduction deficits, morphological and metabolic abnormalities of peripheral nerves (both large motor and sensory fibers and small sensory fibers), the spinal cord, and dorsal root ganglia (DRG), indicating that *ob/ob* mice could be a useful model for studying PDN and chronic pain states associated with morbid obesity similar to those states that occur in humans.

Although the effective treatment of chronic pain remains elusive, recent findings suggest the importance of T-channels in peripheral nociception, raising the possibility that modulation of those channels might be therapeutic in the treatment of acute (6,7) and chronic (8) pain. For example, it has been shown that pharmacological blockade of T-channels with mibefradil and ethosuximide (9), and with (3β,5α,17β)-17-hydroxyestrane-3-carbonitrile (ECN) (10), alleviates mechanical hypersensitivity induced by peripheral nerve injury in rats. Additional evidence regarding the importance of peripheral T-channels in nociception has been provided by the antisense study of Bourinet et al. (11) involving isoform-specific oligonucleotides that downregulate each of the three isoforms of pore-forming subunits of T-channel, $Ca_v3.1$ (α1G), $Ca_v3.2$ (α1H), and $Ca_v3.3$ (α1I), in DRG cells. In this study, only the oligonucleotides downregulating the mRNA of $Ca_v3.2$ were effective in alleviating thermal and mechanical hypersensitivity in the rat model of mononeuropathy. Moreover, $Ca_v3.2$ knockout mice were shown to have decreased responses to acute noxious stimuli (12), further suggesting that T-channels are crucial in nociception.

Furthermore, rats with streptozotocin (STZ)-induced type 1 PDN have significant enhancement of T-current–dependent cellular excitability in acutely dissociated DRG neurons (13). However, behavioral data that would provide a possible mechanistic link between the upregulation of T-currents and symptoms of hyperalgesia in PDN are lacking. Thus, we examined whether these channels are important in the pathophysiology and development of painful PDN in an animal model of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Ethics approval was obtained for all experimental protocols from the University of Virginia Animal Care and Use Committee, Charlottesville, VA. All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the U.S. National Institute of Health. Every effort was made to minimize animal suffering and the number of animals used.

Morbidly obese (*ob/ob*) female mice and their age-matched wild-type counterparts (C57BL/65J), as well as α1H knockout female mice (α1H−/−) and their age-matched littermates (α1H+/+), were studied between the ages of 4 weeks and 30 weeks. It is important to discuss our choice of female mice for this study. Despite the fact that females are more sensitive than males to many pain conditions and that the majority of pain sufferers are women (14–16), less than 8% of currently available animal pain studies include female animals (16). This is in part because studying pain in females is complicated by estrous cycle–dependent variability in nociceptive thresholds. Our behavioral sensory testing of *ob/ob* mice was facilitated by the fact that these mice remain indefinitely prepubertal and without an estrous cycle (17) and was motivated by the fact that there is a significantly higher prevalence of obesity among women than men (18). Furthermore, female mice are easier to handle during behavioral testing. The mice body weights and blood glucose levels were monitored weekly by analyzing tail blood samples using an Accu-Check glucometer (Roche Diagnostics, Indianapolis, IN).

Chemicals. ECN was freshly dissolved in a vehicle containing 15% cyclodextrin solution ([2-hydroxypropyl]-β-cyclodextrin solution; Sigma, St. Louis, MO) and was balanced at pH 7.4 just before injection.

Behavioral testing

Assessment of thermal sensitivity. The paw withdrawal latency (PWL) to thermal stimulation was measured as described previously (19–21) (also see supplemental material, available in an online appendix at http://diabetes.diabetesjournals.org/cgi/content/full/db08-1763/DC1).

Assessment of mechanical sensitivity. The paw withdrawal response (PWR) to mechanical stimulation was measured by our standard method using von Frey filaments (6–8,10,13,22) (supplemental Material). This method was modified from Chaplan et al. (23) to allow time for effective daily assessment of mechanical sensitivity while minimizing residual behavioral responses from repetitious testing (e.g., learning, habituation).

Assessment of sensorimotor abilities. The sensorimotor battery consisted of three tests, the ledge, platform, and inclined screen, as described by Creeley et al. (24) (supplemental material).

Statistical analysis. PWLs and PWRs were subjected to ANOVA containing two within-subject variables: paw (right vs. left) and test session (before the administration of vehicle or test compound vs. each posttreatment time point). Two between-subject variables were the type of mouse (*ob/ob* vs. wild type or α1H−/− vs. α1H+/+) and the age of the mouse. Relevant pairwise comparisons were also done (Holm-Sidak method). α-Levels were adjusted using the Bonferroni procedure when appropriate. All data are expressed as means ± SE. If data did not show normal distribution, we used the Mann-Whitney rank sum test.

The thermal and mechanical hypersensitivity phenotype of *ob/ob* mice in our study was confirmed by three independent examiners. However, because of differences in the size of wild-type and *ob/ob* mice, true blinding was not possible. All drug injections were performed in a blinded manner.

Insulin administration. A group of five *ob/ob* mice received NPH insulin (Eli Lilly and Company, Indianapolis, IN) in two daily intraperitoneal injections from 8 to 12 weeks of age. The total daily dosage ranged from 10 to 40 units and was based on morning glucose values. Control *ob/ob* and wild-type mice received 0.2 ml saline i.p. twice daily.

Morphometry. Distal segments of the sural nerve were dissected, fixed overnight, sectioned, and analyzed as described in supplemental material.

Induction of diabetes. α1H−/− and α1H+/+ animals received a single intraperitoneal injection of 200 mg/kg STZ (Sigma) that had been freshly prepared in saline (13). Mice that did not develop hyperglycemia within 3 days post-STZ were excluded from the study.

Quantitative real-time PCR. Lumbar DRGs or the lumbar spinal cord were dissected and prepared as we described previously (13) (see supplemental material).

Electrophysiological studies. We analyzed data as reported elsewhere (25). For one experiment, we dissected 6–8 lumbar DRGs as previously described (13,26). We focused only on smaller cells having an average soma diameter of 20–30 μm because previous studies have confirmed that most of them are likely to belong to unmyelinated and thinly myelinated polymodal nociceptors in vivo (27,28).

RESULTS

Recent data indicate that *ob/ob* mice develop signs of PDN (4,5) in the setting of transient and mild hyperglycemia along with impaired glucose tolerance, hyperlipidemia, and insulin resistance. Thus, we followed *ob/ob* mice and their wild-type counterparts from 4 to 30 weeks of age (Fig. 1). The body weight of *ob/ob* mice doubled by 10 weeks of age and tripled by 20 weeks as compared with their weight at 4 weeks, although the weight gain in wild-type mice was less than 55% (Fig. 1*A*).

Furthermore, *ob/ob* mice started to develop significant hyperglycemia with blood glucose levels >250 mg/dl (Fig. 1*B*, dashed line) at 7 weeks of age. The most severe hyperglycemia occurred between the ages of 8 and 18 weeks. Although *ob/ob* mice remained morbidly obese, at the age of about 20 weeks they had a spontaneous decrease in blood glucose levels. Based on this observation, for the purpose of all our studies of PDN, we divided *ob/ob* mice into three groups: obese but normoglycemic (4 to 7 weeks old); morbidly obese and hyperglycemic (8 to 20 weeks old, within the rectangle, Fig. 1*A* and *B*); and morbidly obese but with reduced hyperglycemia (>20 weeks old).

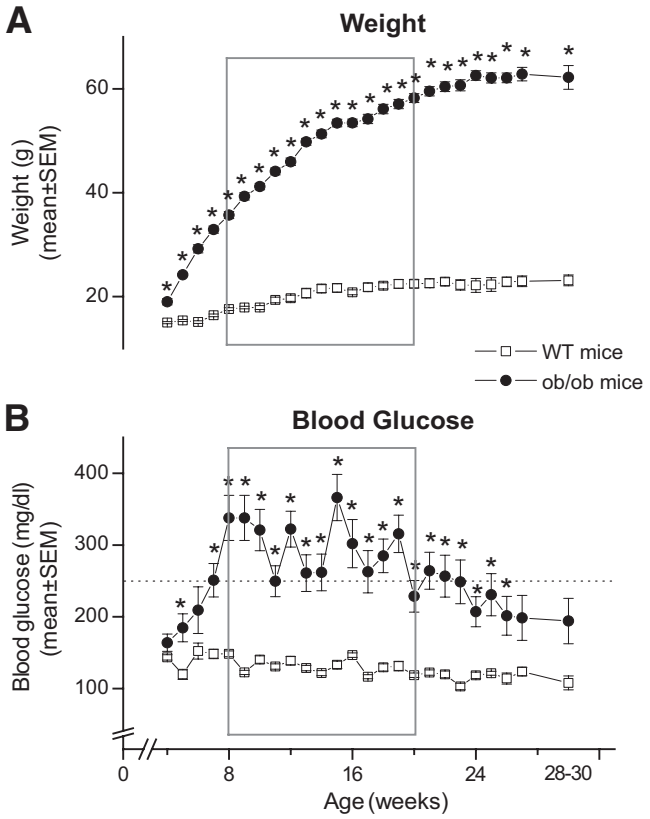

FIG. 1. Age-dependent weight gain (*A*) and blood glucose levels (*B*) in *ob/ob* and wild-type mice. *A*: *Ob/ob* mice (●) rapidly gain weight from the age of 4 weeks and are significantly heavier than age-matched wild-type mice (□) from 4 weeks of age (P* < 0.001). Compared with 4-week-olds, the body weight of *ob/ob* mice doubles around 10 weeks of age and triples around 20 weeks of age, at which point it reaches a plateau. Wild-type mice maintain steady body weight throughout the entire age span (4 to 30 weeks of age) (*n* = 12–24 *ob/ob* mice; *n* = 12–24 wild-type mice). *B*: *Ob/ob* mice develop significant hyperglycemia (blood glucose levels >250 mg/dl) from 7 weeks of age and remain hyperglycemic until 23 weeks of age (●). Spontaneous decrease in blood glucose was recorded after the age of 23 weeks. Blood glucose levels in wild-type matched controls (□) remains steady and within normal limits throughout the entire age span (**P* < 0.05 *ob/ob* vs. wild-type mice). The area within the rectangle indicates ages (8 to 20 weeks old) when *ob/ob* mice are morbidly obese and significantly hyperglycemic (*n* = 12–24 *ob/ob* mice; *n* = 12–24 wild-type mice). WT, wild type.**

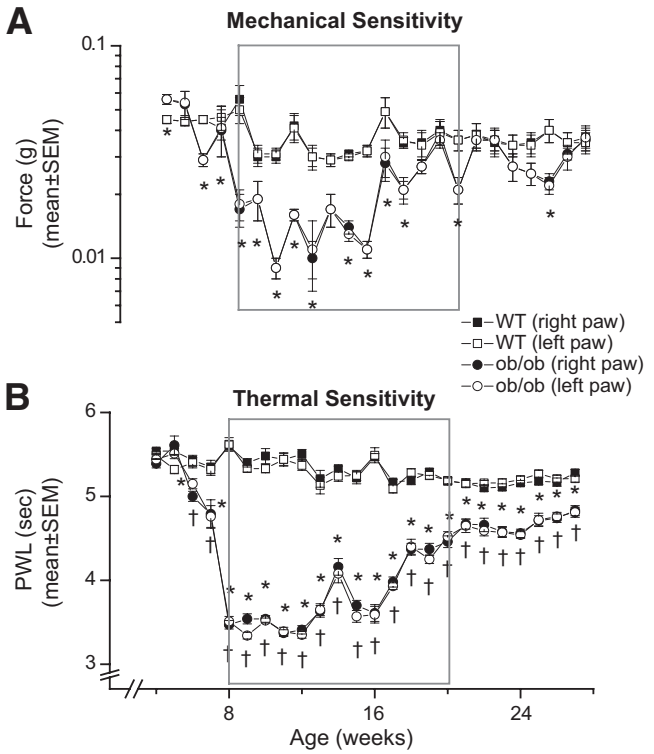

FIG. 2. *Ob/ob* mice demonstrated significant mechanical (*A*) and thermal hypersensitivity (*B*) that coincided with morbid obesity and significant hyperglycemia (boxed area). *A*: *Ob/ob* mice had highly variable but significant mechanical hypersensitivity in both right (●) and left paws (○) (P* < 0.05) as compared with age-matched wild-type mice (right paws, ■; left paws, □). Peak mechanical hypersensitivity occurred from 8 to 16 weeks. *B*: When PWLs were measured, we found significant (**P* < 0.001) decreases in both right (●) and left paws (○) of *ob/ob* mice as compared with age-matched controls. Although that peak decrease was detected from 8 to 16 weeks of age, PWL decreases remained significant as compared with those of wild-type mice throughout the age span. Similarly, there was a significant decrease in thermal PWLs in *ob/ob* mice from 6 weeks of age as compared with their PWLs at 4 weeks of age (†*P* < 0.001) (*n* = 12–18 *ob/ob* mice; *n* = 12–18 wild type mice). WT, wild type.**

To examine the development of abnormal pain sensation, we studied mechanical (Fig. 2*A*) and thermal (heat) sensitivity (Fig. 2*B*) in *ob/ob* mice at 4 to 27 weeks of age and compared the findings to those from age-matched wild-type mice. In Fig. 2, the area within the rectangle highlights the group from 8 to 20 weeks of age, when morbid obesity was accompanied by significant hyperglycemia (as indicated in Fig. 1). Unlike wild-type mice, which had fairly small weekly fluctuations in mechanical sensitivity, *ob/ob* mice started to develop mechanical hypersensitivity at 6 weeks; this sensitivity remained significant until 20 weeks of age (except for 18- and 19-week-old mice) (Fig. 2*A*) at which point it subsided.

Beginning at 6 weeks of age, *ob/ob* mice were also hypersensitive to noxious heat (Fig. 2*B*). Severe thermal hypersensitivity was detected in *ob/ob* mice from 8 until 16 weeks of age, thus coinciding with hyperglycemia.

We considered the possibility that apparent resolution of thermal and mechanical hypersensitivity in older *ob/ob* mice can be a consequence of progressive damage to the peripheral sensory nerves previously exposed to significant hyperglycemia. Thus, we performed a morphometric study of sural nerves in mice 10–12 and 21–22 weeks old using light microscopy. Of the measured parameters, there were no significant differences between age-matched *ob/ob* and wild-type mice in total fiber number, fiber density, fiber area and diameter, and axon area and diameter (n = 4 per group, $P > 0.05$, data not shown). Between 10 and 12 weeks of age when *ob/ob* mice are at the peak of hyperglycemia, they exhibit evidence of peripheral neuropathy manifested as a 19% decrease in myelin thickness and a 34% increase in axon-to-myelin area ratios compared with age-matched wild-type mice (n = 4 per group, $P < 0.05$, data not shown). When myelin thickness and axon-to-myelin area ratios in mice 10–12 weeks old were compared with those in mice 21–22 weeks old, we found no difference between the two age-groups in either parameter. This would suggest that improvement of sensory symptoms in older *ob/ob* mice is less likely to be caused by progressive deterioration of peripheral sensory fibers.

To begin to understand the cellular and molecular mechanisms of hyperalgesia associated with PDN in *ob/ob* mice, we examined the electrophysiological properties of T-currents and analyzed the expression of mRNA for the three isoforms of T-channels in lumbar DRG neurons obtained from *ob/ob* and wild-type mice (Fig. 3). First, we used qRT-PCR to determine the expression of mRNA for three isoforms of T-channels in lumbar DRGs from *ob/ob* mice at 10–12 weeks of age and wild-type controls. We found that the α1H isoform, as compared with α1G and I, was the most prevalent in both wild-type (n = 4) and *ob/ob* mice (n = 3). Importantly, we found that the levels of α1H in lumber DRGs were almost fourfold higher in *ob/ob* mice than wild-type mice ($P < 0.001$, Fig. 3*A*). In contrast, relative expression of mRNA for T-channel isoforms did not statistically differ when compared in lumbar DRGs from *ob/ob* and wild-type mice at age 20–22 weeks (n = 3–5 mice per group, $P > 0.05$, data not shown). In contrast to our findings in DRGs, in corresponding lumbar spinal cord tissue, at age 10–12 weeks, the levels of α1G were not different; α1H and α1I were slightly decreased in *ob/ob* mice (20 and 30%, respectively; n = 3–5 mice per group; $P < 0.05$; data not shown). Similar to the findings in DRGs, there was no significant difference in the levels of mRNA in either isoform in lumbar spinal cord tissues of the wild-type and *ob/ob* groups at the age 20–22 weeks (n = 3–5 mice per group, $P > 0.05$, data not shown).

Representative families of T-currents in DRG cells from wild-type and *ob/ob* mice are shown in Fig. 3*B*, which indicate large enhancement of T-current amplitudes in *ob/ob* mice 10–12 weeks old. To establish the magnitude of the increase and to express it as current density, we normalized maximal peak T-currents to the cell capacitance. The histograms (Fig. 3*C*) indicate that T-current density was enhanced more than twofold in DRG cells from *ob/ob* mice as compared with cells from wild-type mice at the age of 10–12 weeks and 1.5-fold at the age of 16 weeks. However, we did not find a significant difference between the two groups at 5–6 weeks, 18 weeks, or 28–30 weeks of age. Thus, it appears that upregulation of T-currents in DRG neurons coincides with significant hyperglycemia and the development of severe thermal and mechanical hypersensitivity. Furthermore, normalization of T-current density in DRG cells of older *ob/ob* mice correlates well with normalization of message for α1H isoform in DRG tissue homogenates.

Previous in situ hybridization (29) and knockout studies (30) have established that α1H is a predominant isoform of T-channels in DRG cells from normal mice. Thus, we examined the pharmacological properties of these cells in the *ob/ob* model of PDN, testing their sensitivity to nickel,

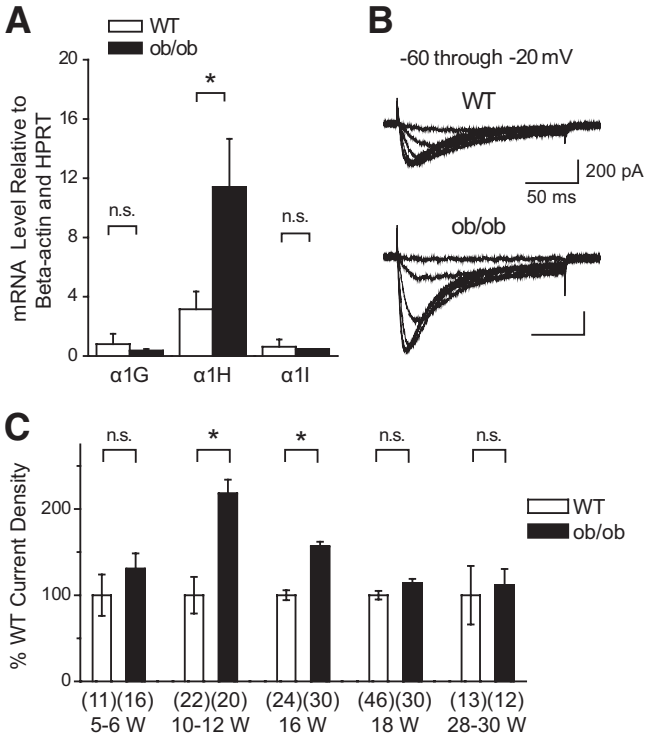

FIG. 3. Upregulation of T-current in smaller DRG cells after the development of hyperglycemia in *ob/ob* mice. *A*: qRT-PCR analysis of the relative expression of mRNA for three isoforms of T-channels in lumbar DRGs and from *ob/ob* mice and age-matched wild-type controls at 10–12 weeks of age. There was a fourfold upregulation of α1H isoform but no significant change in α1G and α1I isoforms in lumbar DRG tissues from *ob/ob* mice compared with wild-type mice (*P < 0.001). *B*: Raw current traces evoked in DRG cells from wild-type (*top panel*) and *ob/ob* (*bottom panel*) mice 10–12 weeks old by voltage steps from −90 mV (V_h [holding potential]) to V_t (test potential) from −60 through −20 mV in 10-mV increments. Calibrations on top bars pertain to both panels. *Ob/ob* mouse had increased absolute amplitude of isolated T-current more than twofold at a range of test potentials from −50 to −20 mV. *C*: Histograms indicate average T-current amplitudes (V_t −30 mV) in DRG cells from wild-type (open bars) and *ob/ob* mice (filled bars) expressed as percent current density normalized to wild type. The size of each sample is in parentheses; vertical bars are SE of multiple determinations. Weeks of age for each of the five groups are indicated at the bottom. In each group, recordings were made from at least three mice. At the peak of hyperglycemia and sensory hypersensitivity (age 10–12 weeks), peak T-current density averaged 20 ± 4 pA/pF in the wild-type group and 44 ± 7 pA/pF in the *ob/ob* group. At 16 weeks of age, T-currents were still significantly upregulated in the *ob/ob* group by about 1.5-fold, with normalization occurring at 18 weeks of age. Before the onset (5–6 weeks) and at the outset of hyperglycemia (28–30 weeks of age), peak T-current density was not different between the wild-type group and the *ob/ob* group (P > 0.05). Asterisk indicates significant value by Mann-Whitney test (P < 0.01); n.s. indicates P > 0.05. HPRT, hypoxanthine-guanine-phosphoribosyltransferase; WT, wild type.

a α1H-specific T-channel blocker (31). The IC_{-50}s for nickel in DRG cells from control mice (25 ± 7 μmol/l, n = 4 cells) versus DRG cells from *ob/ob* mice (16 ± 3 μmol/l, n = 8 cells) were very similar (data not shown).

Because development of sensory hypersensitivity and upregulation of T-current in DRG cells correlates well with hyperglycemia in *ob/ob* mice, we administered insulin daily for 4 weeks (age 8–12 weeks) in *ob/ob* mice and reasoned that reversal of hyperglycemia should abolish or at least diminish hypersensitivity in vivo and upregulation of T-current density in DRG neurons in vitro. Indeed, daily insulin treatments resulted in daily blood glucose levels ≤250 mg/dl (Fig. 4*A*) and almost complete gradual normalization of both thermal (Fig. 4*B*) and mechanical (Fig. 4*C*) hypersensitivity. Importantly, in parallel with the reversal of hyperalgesia, insulin treatments also completely reversed upregulation of T-current density. DRG cells from insulin-treated *ob/ob* mice (12 weeks old) had an averaged current density of 87 ± 7% of that from age-matched wild-type mice (n = 13, P > 0.05, data not shown).

Our previous in vitro studies indicated that ECN is a selective and potent blocker of T-current in DRG cells (32,33) and has potent peripheral analgesic properties in vivo (10,22). For these reasons, we performed dose-response experiments in *ob/ob* mice at the age when they are hypersensitive (10–12 weeks of age, Fig. 2) by systemically injecting ECN (at 5, 10, or 25 mg/kg i.p.). Responses to noxious thermal stimuli were recorded over a 5-h period after the injection (Fig. 5). To confirm the stability of thermal sensation before injection, we compared the mice latency times a couple of days before the dose-response experiment (Fig. *B*, baseline) with the latency obtained immediately before injection (0 min). We found no difference between these recordings for either right (Fig. 5*A* and *C*) or left paws (Fig. 5*B* and *D*), although the baseline in wild-type mice (marked with dotted line) was increased compared with that in *ob/ob* mice. On injection of ECN, we recorded a similar dose-dependent increase in the latency time in both right (Fig. 5*A*) and left hind paws (Fig. 5*B*) of *ob/ob* mice, with the peak effect recorded at 120 min. In contrast, vehicle injection resulted in steady PWL recordings throughout the testing period. The lowest dose of ECN (5 mg/kg) caused small but significant alleviation of hypersensitivity. At 10 mg/kg, ECN was ineffective in wild-type mice (Fig. 5*C* and *D*, circles) but caused a significant increase in PWLs in *ob/ob* mice. The highest dose, 25 mg/kg, although effective in wild-type mice (Fig. 5*C* and *D*), had a much more profound effect in *ob/ob* mice, with sensitivity at a peak (120 min) similar to the baseline sensitivity of wild-type mice (dotted line). This suggested complete, although transient, normalization of thermal hypersensitivity in *ob/ob* mice.

To study the effects of ECN on mechanical sensitivity, we performed a dose-response experiment by determining the PWRs between 90 and 120 min after its injection. The choice of this time was based on the timing of the peak effect on thermal hypersensitivity. Although the lowest dose (5 mg/kg i.p.) had no effect, 10 mg/kg of ECN caused significant alleviation in mechanical hypersensitivity as compared with the vehicle in both right (Fig. 6*A*) and left (Fig. 6*B*) paws. Note that the baseline recordings of PWRs are decreased in wild-type mice (marked with dotted line) (Fig. 6*C*, right paw, and *D*, left paw) and that ECN, at 10 mg/kg, had no effect on mechanical sensitivity in wild-type mice. The highest dose of ECN, 25 mg/kg, caused a significant decrease (~40%) in the PWRs, resulting in complete reversal of mechanical hypersensitivity in *ob/ob* mice.

To determine specificity of ECN in vivo in diabetic animals, we performed a series of experiments with STZ-injected α1H knockout mice (Fig. 7*A*), focusing on the most effective dose of ECN (25 mg/kg i.p.) and using thermal (Fig. 7*B* and *C*) and mechanical (Fig. 7*D* and *E*) sensory testing. STZ-injected α1H−/− and age-matched α1H+/+ mice developed hyperglycemia with blood glucose levels above 400 mg/dl to similar degrees (Fig. 7*A*, *insert*). Interestingly, α1H−/− mice did not develop thermal hypersensitivity as evidenced with stable PWLs in both right and left paws for 4 weeks after injections of

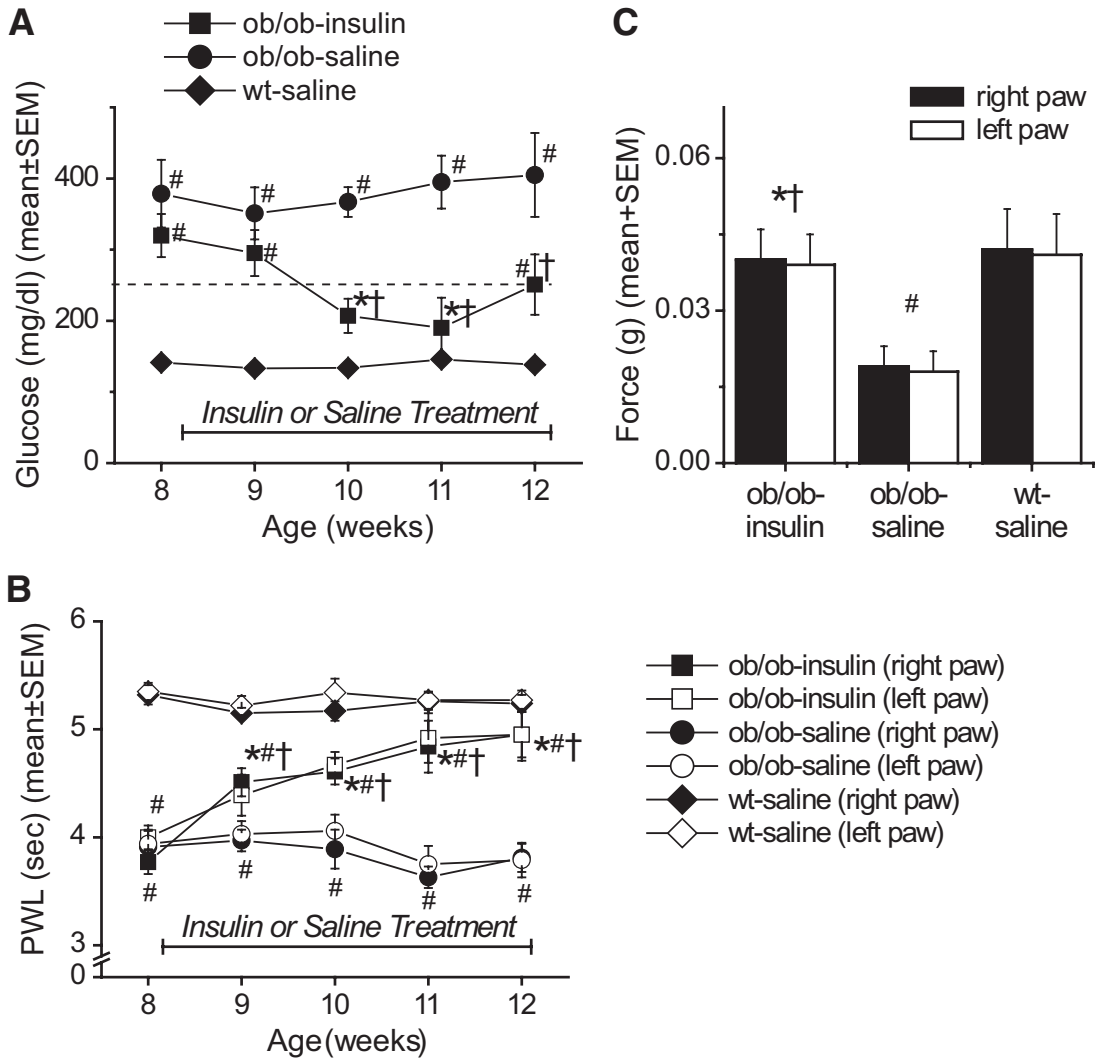

FIG. 4. Insulin treatment alleviates heat and mechanical hypersensitivity in *ob/ob* mice. Beginning at 8 weeks of age, *ob/ob* and wild-type mice were treated with intraperitoneal insulin or saline twice daily. *A*: Insulin-treated *ob/ob* mice (■) had reduced blood glucose values by 10 weeks of age compared with saline-treated *ob/ob* mice (†$P < 0.001$) and to original 8-week levels (*$P < 0.01$). Glucose levels in *ob/ob* saline-treated mice (●) were greater than wild-type saline-treated mice (♦) throughout the testing period, while *ob/ob* insulin-treated mice were only different than wild-type saline-treated mice during select weeks (#$P < 0.05$). *B*: Hypersensitivity in *ob/ob* insulin-treated mice progressively improved compared with 8-week values (*$P < 0.001$) and with *ob/ob* saline-treated mice (†$P < 0.001$); however, PWLs remained significantly less than wild-type saline-treated mice (#$P < 0.05$). *C*: At 12 weeks of age, mechanical thresholds in *ob/ob* insulin-treated mice were significantly greater than 8-week values (*$P < 0.01$) and *ob/ob* saline-treated mice (*$P < 0.05$). *ob/ob* saline-treated mice remained hypersensitive as compared with wild-type saline-treated mice (#$P < 0.05$) ($n = 5$ *ob/ob* mice per group; $n = 6$ wild-type mice). WT, wild type.

STZ. In contrast, wild-type littermates developed thermal hyperalgesia with maximal decrease in PWLs of about 30% at 1–2 weeks after injections of STZ (Fig. 7*A*). We next found that ECN caused a significant increase in PWLs in both paws of diabetic α1H+/+ littermates (Fig. 7*B*). Vehicle injection caused no changes in PWLs throughout the testing interval (data not shown, $n = 5$ animals). In contrast, in α1H−/− diabetic mice (Fig. 7*C*), ECN had no effect on PWLs in either paw. Similarly, when the effect of ECN on mechanical sensitivity was tested between 90 and 120 min, ECN caused a significant decrease in mechanical hypersensitivity in diabetic α1H+/+ mice (Fig. 7*D*) but a complete lack of effect in diabetic α1H−/− mice (Fig. 7*E*) in both paws. Similar to absence of the thermal hypersensitivity, diabetic α1H−/− mice did not develop mechanical hypersensitivity because PWRs were not different from healthy wild-type mice (data not shown). In summary, these data strongly suggest that α1H channel is required for the development of early painful PDN in a common model of STZ-induced diabetes and that diabetic α1H−/− mice are insensitive to the analgesic effects of ECN.

We also considered the possibility that ECN might nonspecifically decrease thermal and mechanical sensation by inducing a general depression of behavioral performance. To determine whether ECN, given at its maximally effective analgesic dose of 25 mg/kg, causes sensorimotor disturbances such as motor weakness or sedation, which could affect the validity of behavioral sensory testing, we did a battery of sensorimotor tests focused on agility and fine motor abilities (Fig. 8). Wild-type ($n = 5$) and *ob/ob* ($n = 5$) mice at 10–12 weeks of age were tested using an inclined plane (Fig. 8*A*), platform (Fig. 8*B*), and ledge (Fig. 8*C*) before ECN injection and at 90–120 min after injection (at approximately the peak of the effect of ECN on mechanical and thermal sensation). The responses of ECN-treated animals did not significantly differ from those responses before injection on any of these tests, suggesting that

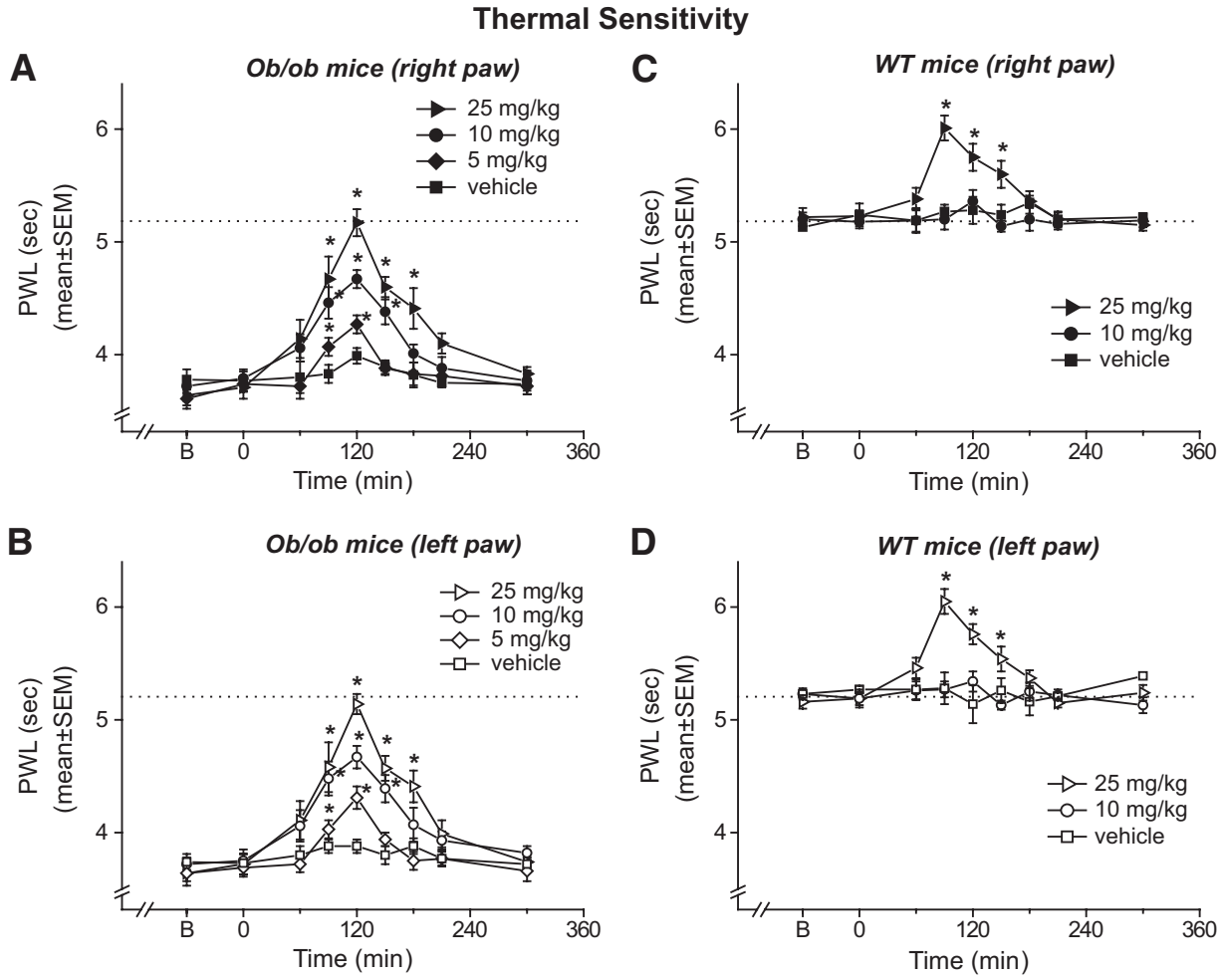

FIG. 5. ECN, a selective T-channel blocker and neuroactive steroid, induced greater dose-dependent alleviation of heat hypersensitivity in *ob/ob* mice (*A* and *B*) than in wild-type mice (*C* and *D*). For all dose-response experiments, 10- to 12-week-old mice (at the peak of thermal hypersensitivity) were used. ECN (5 mg/kg i.p.) caused small but significant increases in PWLs in right (*A*) and left paws (*B*) of *ob/ob* mice, with the peak effect at 120 min after injection (P* < 0.001). When 10 mg/kg of ECN was administered, the peak effect still occurred at 120 min but lasted longer; that is, the increase in PWLs was still significant at 150 min compared with those at 0 min (immediately before the injection) (**P* < 0.001) in both right (*A*) and left paws (*B*). Injection of 25 mg/kg of ECN caused a profound increase in PWLs that remained significant 180 min after injection (**P* < 0.001). At the peak effect, the highest dose of ECN caused complete reversal of mechanical hypersensitivity; that is, PWLs were similar to the baseline PWLs recorded in wild-type mice (dotted line). When ECN was administered to wild-type mice at 10 mg/kg, a dose that was effective in *ob/ob* mice, there was no effect on PWLs at any time in either right (*C*) or left paws (*D*); that is, PWLs remained at the baseline level (dotted line). Only at 25 mg/kg did ECN cause a significant increase in PWLs in wild-type mice, with a peak effect at 90 min. However, this effect was not as robust as that in *ob/ob* mice (**P* < 0.001) (*n* = 6 *ob/ob* mice per group; *n* = 6 wild-type mice per group). WT, wild type.**

the effect of ECN on alleviation of neuropathic thermal and mechanical hypersensitivity is most likely mediated by T-channels located in the pain pathways.

DISCUSSION

A major finding of our study is that PDN in hyperglycemic *ob/ob* mice is accompanied by pathophysiological disturbances in the function of T-channels in sensory neurons. In particular, we have shown a large increase in the amplitude of T-currents, with the expression of α1H mRNA being most affected. T-channel upregulation coincided with behavioral disturbances that usually are indicative of painful PDN, measured as thermal and mechanical hypersensitivity. Both types of hypersensitivity were dose-dependently alleviated by ECN, a neuroactive steroid and selective T-channel antagonist. Based on our behavioral, biochemical, and biophysical evidence, we propose that T-channels potentially are important therapeutic targets for use in the management of painful PDN.

Clinical reports regarding the severity of PDN symptoms suggest a direct correlation between hyperglycemia and a propensity for neuropathic pain (NPP)-like pathology, recommending strict glycemic control as one of the mainstays of therapy (34). Our findings with morbidly obese *ob/ob* mice confirm that two important signs of NPP, thermal and mechanical hypersensitivity, coincide with hyperglycemia; that is, hypersensitivity was most profound in mice between the ages of 8 and 16 weeks and the improvement of hyperglycemia with insulin alleviated these hypersensitivities. The lack of mechanical hypersensitivity and decrease in thermal hypersensitivity at ages later than 20 weeks could be attributed to reduction of hyperglycemia. However, more permanent changes in axonal physiology, metabolism, and morphology, including decreased nerve conduction velocity, demyelination, impaired axonal transport, and axonal atrophy, could also be implicated because they play an important role in hyposensitivity in later stages of other PDNs (35). Inter-

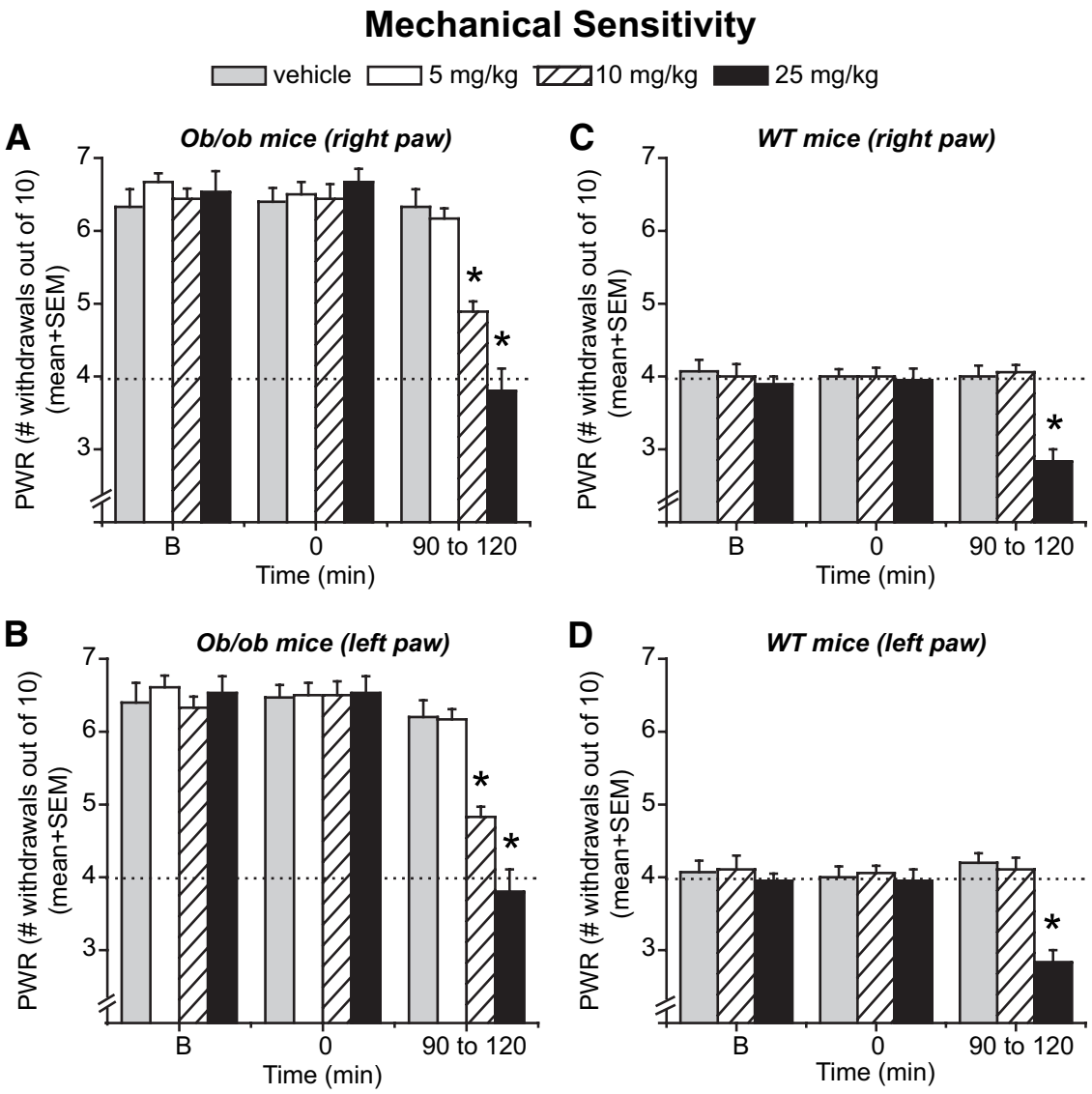

FIG. 6. ECN, a selective T-channel blocker and neuroactive steroid, induced greater dose-dependent alleviation of mechanical hypersensitivity in *ob/ob* mice (*A* and *B*) than in wild-type (*C* and *D*) mice. All dose-response experiments were done in 10- to 12-week-old mice (at the peak of mechanical hypersensitivity). PWRs were recorded at 90–120 min after injection of ECN (at the peak effect on thermal hypersensitivity). Intraperitoneal injection of 5 mg/kg of ECN had no effect on PWRs in either right (*A*) or left paws (*B*) of *ob/ob* mice. When 10 mg/kg was given, PWRs were significantly decreased compared with those at 0 min (immediately before the injection) (*$P < 0.001$) in both right (*A*) and left paws (*B*). The highest dose of ECN, 25 mg/kg, caused a profound decrease in PWRs leading to complete reversal of mechanical hypersensitivity; that is, PWRs were similar to the baseline PWRs recorded in wild-type mice (dotted line). When 10 mg/kg of ECN was administered to wild-type mice, there was no effect on PWRs in either right (*C*) or left paws (*D*); that is, PWRs remained at the baseline level (dotted line). At the highest dose, 25 mg/kg, ECN caused a significant decrease in PWRs (*$P < 0.001$ compared with vehicle at 90–120 min) ($n = 6$ *ob/ob* mice per group; $n = 5–6$ wild-type mice per group). WT, wild type.

estingly, we found no evidence of progression of peripheral nerve damage in *ob/ob* mice using sural nerve morphometry. However, additional morphometric studies are needed before conclusively determining whether *ob/ob* mice fully recover from hyperglycemia.

In our experiments, thermal and mechanical hypersensitivity in *ob/ob* mice developed in parallel with hyperglycemia and was readily reversed with insulin therapy. However, another recent study also reported mechanical hypersensitivity but thermal hyposensitivity of *ob/ob* mice (5). No reason for this discrepancy is immediately obvious, but possibilities include internal biological variability within the *ob/ob* mice phenotype; different environmental, nutritional, and housing conditions; and a difference in the sensitivity of methods used to measure thermal sensation. In addition, our study focused on female mice, but it is not clear what the sex was in the study by Drel et al. Similarly, variations in the occurrence, duration, modality, and intensity of pain symptoms are well documented among humans with PDN (3,36–38). Nevertheless, our promising findings regarding ECN-induced alleviation of mechanical and thermal hypersensitivity in hyperglycemic *ob/ob* mice suggest that pharmacological antagonism of T-channels in sensory neurons may offer a great advantage in the treatment of PDN, despite poor control of diabetes.

To the best of our knowledge, this is the first report of a nociceptive ion channel alteration in PDN associated with morbid obesity. Although an earlier study reported the upregulation of both T-type and high voltage–activated (HVA) Ca^{2+} currents in small DRG cells in a rat model of type 1 PDN (39), the importance of T-channels was not further studied. Because our study was not designed to

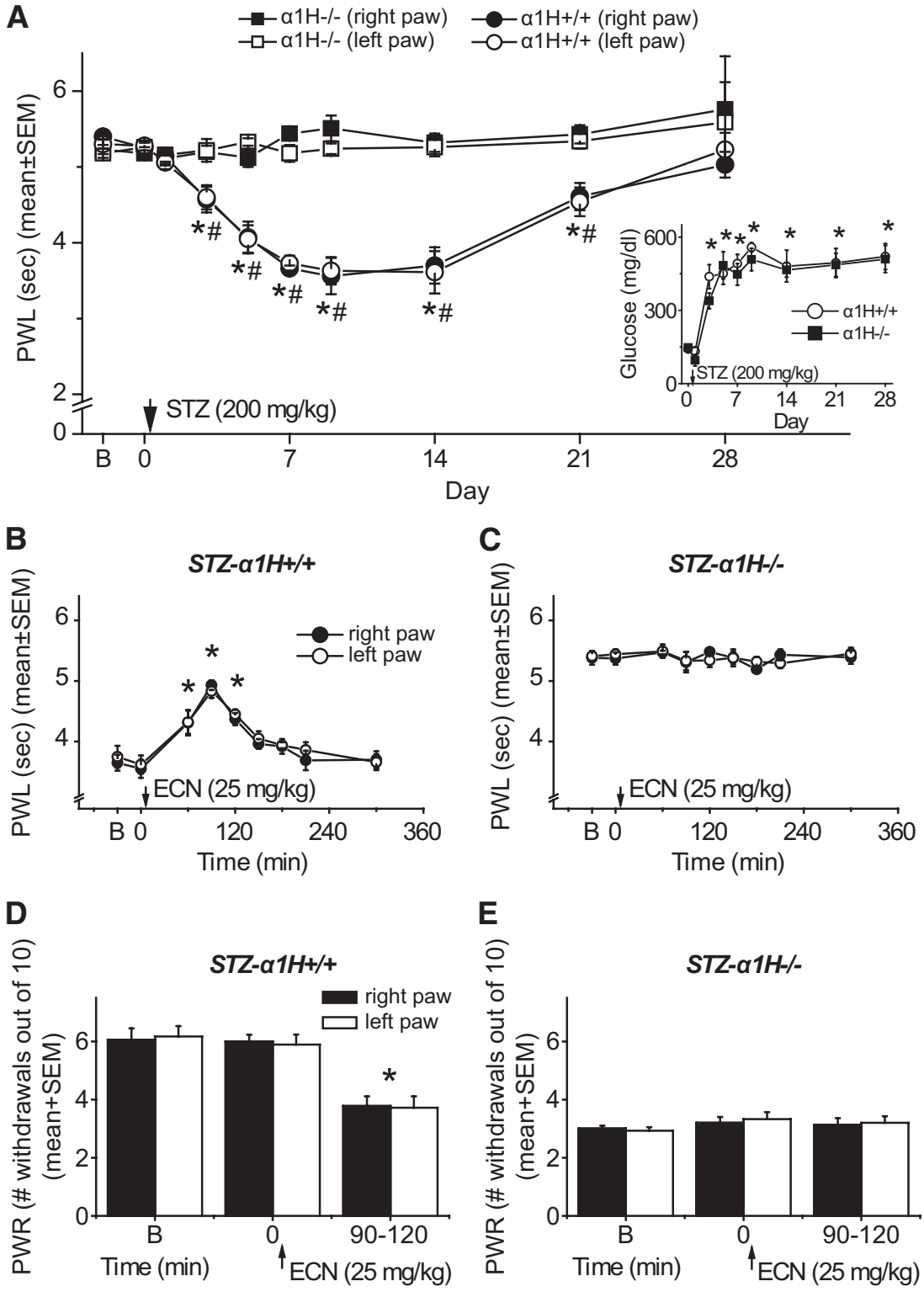

FIG. 7. Diabetic α1H−/− mice do not develop early hyperalgesia and are resistant to the effects of ECN in tests of mechanical and thermal sensitivity. *A*: When 200 mg/kg STZ was administrated intraperitoneally to α1H+/+ mice (circles) and α1H−/− mice (squares), PWLs for the first 4 weeks post-STZ were significantly different between the two groups (#$P < 0.001$). Compared with their initial baselines, only α1H+/+ developed heat hypersensitivity (*$P < 0.01$). Both groups developed hyperglycemia to a similar extent (see insert) with blood glucose greater than initial day 0 values (*$P < 0.001$) ($n = 5$ α1H−/− mice per group; $n = 7$ α1H+/+ mice per group). *B*: When 25 mg/kg of ECN was administered intraperitoneally to diabetic α1H+/+ mice (1–2 weeks after STZ treatment), there was, between 60 and 120 min after ECN injection, a significant increase in PWLs in both right (●) and left paws (○) as compared with PWLs at 0 min (immediately before injection) (*$P < 0.001$). *C*: The same dose of ECN had no effect on thermal sensitivity in diabetic α1H−/− mice (also 1–2 weeks after STZ) throughout the testing period ($n = 5$ α1H−/− mice per group; $n = 6$ α1H+/+ mice per group). *D* and *E*: When mechanical sensitivity was tested at 90 to 120 min after injection of 25 mg/kg of ECN, there was a significant decrease in PWRs in diabetic α1H +/+ mice as compared with those at 0 min (panel D) (*$P < 0.001$) but no effect on PWRs of diabetic α1H−/− mice (*E*) ($n = 5$ α1H−/− mice per group; $n = 6$ α1H+/+ mice per group).

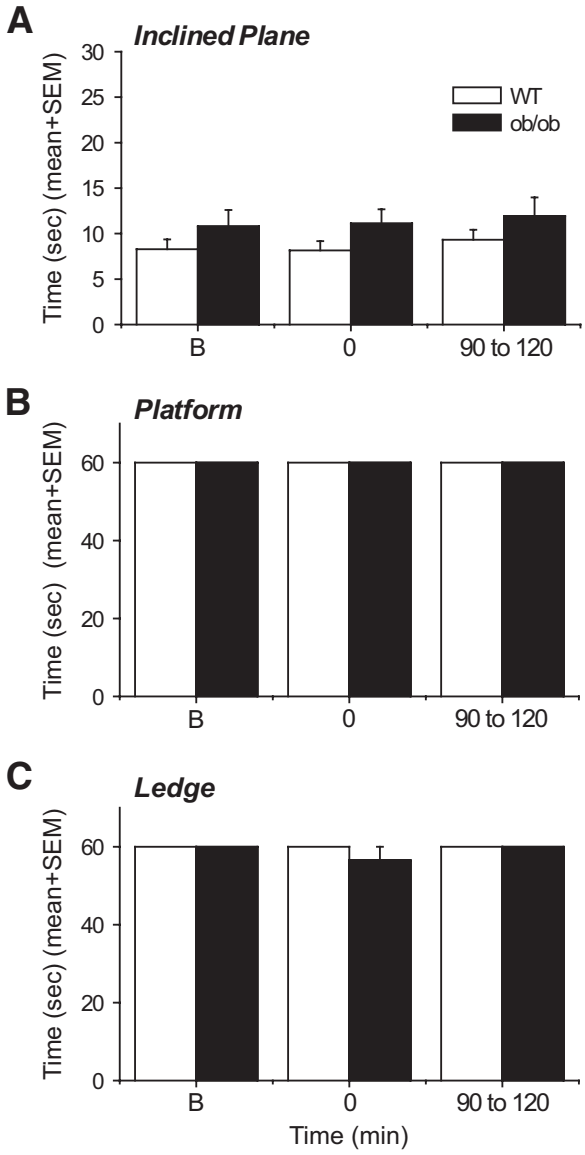

FIG. 8. Lack of effect of ECN on sensorimotor tests in *ob/ob* and wild-type mice. Panels show histograms of average time in seconds in different sensorimotor tests such as inclined plane (*A*), platform (*B*), and ledge (*C*) for wild-type (open bars) and *ob/ob* mice (filled bars). Baseline measurements were taken 2 days before (*B*), immediately before (0 min), and 90–120 min after ECN injection (25 mg/kg i.p.). Vertical bars indicate SE of multiple determinations. We used two-way ANOVA followed by pairwise comparison to analyze results, finding that ECN had no significant effect on either test, because $P > 0.05$ in comparisons of time points within each animal group ($n = 5$ mice in each group). WT, wild type.

examine the importance of other voltage-gated channels, we cannot comment on their function in PDN in *ob/ob* mice. However, based on our in vivo studies with ECN, HVA Ca^{2+} current appears to be an unlikely target for ECN because this blocker has weak affinity for HVA channels (32). In addition, the lack of any effect of ECN on mechanical and thermal sensation in diabetic α1H$-/-$ mice points to T-channels as the main target of ECN analgesic action. Although a causal correlation among the upregulation of T-currents, enhanced excitability of sensory neurons, and nociception remains to be confirmed, it is generally accepted that the hyperexcitability of sensory neurons contributes to allodynia and hyperalgesia, hallmarks of chronic NPP (40). In that case, T-channel blockade is a potential therapeutic approach to the treatment of NPP. However, based on previously published findings (10) and the data presented here, it appears that, although significant, the alleviation of NPP with ECN is transient. Therefore, synthesis of ECN-like compounds with a longer effect or a slow-release formulation of ECN would be beneficial when repeated administration was indicated.

Possible sites of the analgesic action of ECN other than the DRG neurons remain to be determined. For example, being a small lipophilic molecule, ECN could easily cross the blood-brain barrier to modulate T-channels located in the dorsal horn neurons, an important pain-processing region in the central nervous system (41). However, this appears less likely given that our qRT-PCR data show that T-channel isoforms in the spinal cord of *ob/ob* mice are either not changed (α1G) or are already decreased (α1I and α1H), possibly as a compensatory mechanism in response to diabetes-induced peripheral sensitization to sensory stimuli. Further studies of ECN pharmacokinetics will be needed to address this issue.

In conclusion, we have shown that morbidly obese, hyperglycemic *ob/ob* mice develop thermal and mechanical hypersensitivity, which usually is indicative of painful PDN. Their hypersensitivity is accompanied by biochemical and biophysical modulations of T-channels in DRG neurons, suggesting that T-channels potentially are a novel target for treatment of pain in patients with PDN.

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No potential conflicts of interest relevant to this article were reported.

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