Glycogen synthase kinase-3 is essential for β-arrestin-2 complex formation and lithium-sensitive behaviors in mice

W. Timothy O’Brien,1 Jian Huang,1 Roberto Buccafusca,2 Julie Garstkof,1 Alexander J. Valvezan,3 Gerard T. Berry,2 and Peter S. Klein1,3

1Department of Medicine, Hematology-Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.
2Division of Genetics and The Manton Center for Orphan Disease Research, Children’s Hospital Boston, Harvard Medical School, Boston, Massachusetts, USA.
3Cell and Molecular Biology Graduate Group, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA.

Lithium is the first-line therapy for bipolar disorder. However, its therapeutic target remains controversial. Candidates include inositol monophosphatases, glycogen synthase kinase-3 (GSK-3), and a β-arrestin-2/AKT/protein phosphatase 2A (β-arrestin-2/AKT/PP2A) complex that is known to be required for lithium-sensitive behaviors. Defining the direct target(s) is critical for the development of new therapies and for elucidating the molecular pathogenesis of this major psychiatric disorder. Here, we show what we believe to be a new link between GSK-3 and the β-arrestin-2 complex in mice and propose an integrated mechanism that accounts for the effects of lithium on multiple behaviors. GSK-3β (Gsk3b) overexpression reversed behavioral defects observed in lithium-treated mice and similar behaviors observed in Gsk3b+/− mice. Furthermore, immunoprecipitation of striatal tissue from WT mice revealed that lithium disrupted the β-arrestin-2/Akt/PP2A complex by directly inhibiting GSK-3. GSK-3 inhibitors or loss of one copy of the Gsk3b gene reduced β-arrestin-2/Akt/PP2A complex formation in mice, while overexpression of Gsk3b restored complex formation in lithium-treated mice. Thus, GSK-3 regulates the stability of the β-arrestin-2/Akt/PP2A complex, and lithium disrupts the complex through direct inhibition of GSK-3. We believe these findings reveal a new role for GSK-3 within the β-arrestin complex and demonstrate that GSK-3 is a critical target of lithium in mammalian behaviors.

Introduction
Bipolar disorder (BPD) is a potentially devastating affective disorder affecting 1%–2% of the population worldwide (1). Lithium has remained the first line of therapy for decades (2–4), and yet the molecular target of lithium in BPD remains highly controversial (5). The leading candidates under active investigation as potential therapeutic targets of lithium include inositol monophosphatases and related phosphomonoesterases (6–11), glycogen synthase kinase-3 (GSK-3) (12), and the scaffolding function of β-arrestin-2 (13).

GSK-3 has emerged as a strong candidate for the relevant in vivo target of lithium. Lithium inhibits GSK-3 in vitro (12) and in vivo at therapeutically relevant concentrations (14–20). Inhibition of GSK-3 provides a compelling explanation for the effects of lithium on the development of diverse organisms (8), and these observations are supported by pharmacologic (21–23) and genetic loss-of-function approaches that closely mimic lithium effects on development (8, 24–29). Systemic lithium inhibits GSK-3 in the mouse brain (16, 20, 30, 31) and in peripheral blood cells of patients with BPD (32, 33). Genetic loss of function and inhibition of GSK-3 with structurally diverse inhibitors also parallel lithium effects in diverse behaviors in rodents (13, 20, 34–37). These correlations support GSK-3 as the relevant target of lithium in mammalian behavior.

However, the specificity of lithium action in these settings has not been assessed by rescue experiments to demonstrate a causal role for GSK-3. Furthermore, elegant recent work suggests that behavioral effects of lithium arise through inhibition of the scaffolding function of β-arrestin-2 that mediates interaction of Akt and protein phosphatase 2A (PP2A) to regulate Akt (acting upstream of GSK-3) (13, 38). To test whether lithium-mediated behavioral effects are specifically due to inhibition of GSK-3, we have generated transgenic mice that overexpress GSK-3β (Gsk3b) in the brain and show that Gsk3b overexpression reverses the effects of lithium in multiple behaviors. Using Gsk3b gain-of-function and loss-of-function approaches, we also show that GSK-3 is required for stability of the β-arrestin-2/Akt/PP2A complex and that direct inhibition of GSK-3 disrupts the β-arrestin-2 complex in vivo. Taken together, these findings reconcile 2 leading hypotheses for lithium action and provide strong support for GSK-3 as an essential direct target of lithium in mammalian behaviors.

Results
Overexpression of Gsk3b reverses lithium-sensitive behaviors. Rescue of a gene knockout or drug-induced phenotype by restoring activity of the putative target provides strong support for the hypothesis that the phenotype is due to specific inhibition of the target. This is considered an essential control in the genetic analysis of model organisms, such as yeast and fruit flies, although it is less often applied in mammalian systems. We therefore tested whether transgenic expression of Gsk3b in the brain would reverse lithium-sensitive phenotypes, including established lithium-sensitive behaviors (20) and assembly of the β-arrestin-2/Akt/PP2A complex (13). Initially, we tested whether increasing GSK-3 levels would reverse inhibition of substrate phosphorylation in the presence of lithium chloride (LiCl) in vitro. Raising the overall level of GSK-3 protein by 50% restored substrate phosphorylation in the...
Expression of Gsk3b-his in mouse brain. (A) Schematic of the Gsk3b-his transgene inserted downstream of MoPrp (MoPrp.xho). ex1, exon 1; utr, untranslated region. (B) GSK-3β-his protein expression in cortex, striatum, and hypothalamus of WT, PrpGsk3bL56, and PrpGsk3bL64 transgenic mice was assessed by immunoblotting for 6X-his tag. Partial N-terminal proteolysis yielded a second, smaller band that reacted with C-terminal GSK-3 antibodies and was also observed for endogenous GSK-3β (see D), as reported previously (40, 41). GAPDH was used as the loading control. (C) Total GSK-3β protein expression in cortex and striatum of WT, PrpGsk3bL56, and PrpGsk3bL64 mice detected with an N-terminal, GSK-3β-specific antibody. (D) GSK-3α and GSK-3β protein expression in cortex and striatum, detected with an antibody that recognizes the C-termini of both GSK-3α and GSK-3β antibody, confirms partial proteolysis of endogenous GSK-3β and GSK-3β-his (asterisks). An alternatively spliced form of endogenous GSK-3β is indicated (GSK-3βalt). GSK-3β-his migrates between the endogenous GSK-3α and GSK-3βalt forms.

Figure 1

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presence of LiCl to that of control levels in vitro (Supplemental Results and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI45194DS1). Gsk3b overexpression also restored lithium-inhibited phosphorylation of the endogenous GSK-3 substrate glycogen synthase in human embryonic kidney cells (Supplemental Results and Supplemental Figure 1B) and reversed activation of a Wnt-dependent transcription reporter (similar to TOPFLASH) that was robustly activated by lithium (Supplemental Results and Supplemental Figure 1C). Thus, increasing GSK-3 levels reversed lithium effects in vitro and in cultured cells. We next tested whether transgenic expression of Gsk3b in the brain rescues lithium-sensitive behaviors in mice.

Chronic lithium treatment reduces immobility in the forced swim test (FST), reduces exploratory behavior, and increases time in the open area of the elevated zero maze (EZM) (20). These parallels between lithium action and Gsk3b loss of function are consistent with the hypothesis that Gsk3b is the relevant target of lithium in these behaviors, but to establish a causal role for inhibition of GSK-3 by lithium, we tested whether overexpression of Gsk3b would reverse the behavioral effects of lithium. The mouse prion promoter (MoPrp) drives transgene expression in neurons and glia through-out the central nervous system of mice (39). This promoter was used to drive expression of Gsk3b with a C-terminal 6X-his tag (Figure 1A). Two transgenic founder lines were obtained (PrpGsk3bL56 and PrpGsk3bL64) and were backcrossed into the C57BL/6 background. Expression of the transgene in cortex, hippocampus, amygdala, hypothalamus, striatum, nucleus accumbens, and cerebellum was confirmed by RT-PCR (data not shown) and by Western blot. Gsk3b overexpression also restored lithium-inhibited phosphorylation of endogenous GSK-3β in lysates prepared from striatum and cortex (Supplemental Methods, Supplemental Results, and Supplemental Figure 5), and the general state of Gsk3b transgenic mice were indistinguishable from those of their WT littermates, indicating that Gsk3b overexpression rescues this effect of lithium (Figure 2A). There was no significant effect of Gsk3b overexpression in PrpGsk3bL44 mice, which may reflect the relatively lower level of GSK-3β-his expression in these mice (Figure 1B).
The time spent in the open versus the closed arms is measured. As shown previously (20), lithium treatment increased time in the open areas, and overexpression of \textit{Gsk3b} reversed this effect in both transgenic lines (P < 0.05), restoring time spent in the open area to that of WT control levels (Figure 2B). These observations are consistent with and complementary to the recent observation that knockin of phosphorylation-defective forms of GSK-3\textit{a} and GSK-3\textit{b}, which prevents inhibitory phosphorylation at N-terminal serines, increases time in the open area of the elevated plus maze (33).

Hole poke exploratory behavior measures the frequency at which mice explore holes in the floor of an observation chamber. Chronic lithium reduces the number of hole pokes without affecting overall activity (20). Consistent with previous findings, lithium reduced hole pokes in WT mice (P < 0.05). Overexpression of \textit{Gsk3b} restored the number of hole pokes in lithium-treated \textit{PrpGsk3b\textit{L56}} mice to those of WT control levels (Figure 2C); lithium-treated \textit{PrpGsk3b\textit{L64}} mice also showed an increase in the number of hole pokes that approached but did not achieve statistical significance, which may reflect the lower expression of GSK-3\textit{b}-his in this line. To rule out an effect of overt changes in the state of the mice that could confound the exploratory behavior assessment, data for total activity were collected in the hole board arena. No significant differences were found among the WT, WT with lithium, \textit{PrpGsk3b}, and \textit{PrpGsk3b} with lithium groups (Figure 2D).

In the EZM, mice are placed on a circular track with 2 symmetrically opposed open and enclosed areas, and the time spent in the open versus the closed arms is measured. As shown previously (20), lithium treatment increased time in the open areas, and overexpression of \textit{Gsk3b} reversed this effect in both transgenic lines (P < 0.05), restoring time spent in the open area to that of WT control levels (Figure 2B). These observations are consistent with and complementary to the recent observation that knockin of phosphorylation-defective forms of GSK-3\textit{a} and GSK-3\textit{b}, which prevents inhibitory phosphorylation at N-terminal serines, increases time in the open area of the elevated plus maze (33).

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Heterozygous loss of \textit{Gsk3b} causes behavioral defects that mimic lithium action, including reduced time immobile in the FST and
with ANOVA with Dunn’s post-hoc test, although a 1-tailed Student’s t-test comparing WT and PrpGsk3b+–/– mice showed $P = 0.006$. Reduced exploratory behavior (20, 43); we found that transgenic expression of Gsk3b in Gsk3b+–/– heterozygotes reversed these behavioral defects, demonstrating that the behavioral deficits observed in Gsk3b+–/– mice are also specifically due to Gsk3b loss of function (Supplemental Results and Supplemental Figure 4).

Gsk3b is required for the β-arrestin-2/Akt/PP2A complex. Previous work has defined a central role for β-arrestin-2 as a molecular scaffold that binds Akt and PP2A in neurons of the striatum to mediate dephosphorylation of Akt and, indirectly, reactivation of GSK-3 (13). Lithium also inhibits the in vitro interaction of recombinant β-arrestin-2 and Akt, although at a relatively high concentration of lithium (50 mM). Earlier work has shown that Gsk3b is also a component of the complex (44); we therefore tested whether GSK-3 contributes to the stability of the β-arrestin-2 complex. We isolated striata from WT mice, immunoprecipitated Akt, and blotted for components of the complex. Under basal conditions, Akt, PP2A, β-arrestin-2, GSK-3α, and GSK-3β interacted in the striatum, and association of Akt, PP2A, and β-arrestin-2 was disrupted by LiCl (Figure 3, A–C), as previously published (13, 44, 45). If GSK-3 stabilizes this complex, then structurally distinct GSK-3 inhibitors should mimic lithium and disrupt the complex. Indeed, addition of the GSK-3 inhibitors 6BIO (Figure 3A) or AR-A014418 (Supplemental Results and Supplemental Figure 6) to the immunoprecipitations disrupted interaction of Akt with β-arrestin-2 and PP2A (Figure 3A), similar to LiCl. We also note that the level of GSK-3α/β in the Akt immunoprecipitates is not reduced upon inhibition, perhaps reflecting additional Akt/GSK-3–containing complexes that are not sensitive to GSK-3 inhibition (46–50). To confirm that the β-arrestin-2 complex is sensitive to GSK-3 inhibitors, we performed immunoprecipitations from striatum using an anti–β-arrestin-2 antibody. Consistent with Figure 3A and prior reports, β-arrestin-2 interacted with PP2A (13, 44, 45), and addition of lithium or 6BIO disrupted this interaction (Figure 3B). The β-arrestin-2 immunoprecipitates also contained Akt and GSK-3α/β, but we did not observe dissociation of Akt or GSK-3 when GSK-3 was inhibited; while the reasons for this are unclear, each of these signaling molecules are known to associate in multiple and distinct complexes (46–50), and the β-arrestin antibody used here may identify additional complexes that are not sensitive to GSK-3 inhibition.

These observations suggest that GSK-3 contributes to β-arrestin-2 complex stability and that lithium disrupts the β-arrestin scaffold in vivo by inhibiting GSK-3, in addition to the reported direct effect on β-arrestin-2/Akt interaction. To investigate whether GSK-3 regulates the β-arrestin-2 complex in vivo, we also tested complex formation in the striatum of Gsk3b+–/– mice and found reduced interaction of Akt and PP2A in mice lacking one copy of Gsk3b (Figure 3, C and D). WT and PrpGsk3b+–/– mice were also treated for 12 days with LiCl, as above. Chronic LiCl reduced interaction between Akt and PP2A in WT mice, as reported previously (13), and this was restored in PrpGsk3b+–/– mice (Figure 3, C and D). Taken together, these observations show that the β-arrestin-2/Akt/PP2A/GSK-3 complex requires GSK-3, as both pharmacologic and genetic interference with Gsk3b disrupts complex formation and, importantly, overexpression of Gsk3b restores complex formation in parallel with the rescue of lithium-sensitive behaviors.

**Discussion**

Defining the direct molecular target of lithium is critically important for the development of new drugs to treat BPD and to define the molecular pathogenesis of this common psychiatric disease. We have proposed a set of criteria to validate a putative target of lithium that can be applied in each lithium-sensitive context (5), including (a) in vivo inhibition, (b) pharmacologic...
evidence that structurally diverse inhibitors mimic lithium, (c) genetic evidence through mutation of the target, and (d) reversal of lithium-sensitive behaviors by restoring activity of the target in the presence of lithium.

Each of these criteria is now fulfilled for GSK-3: GSK-3 is inhibited by lithium in the rodent brain (16, 20, 30, 31); diverse GSK-3 inhibitors mimic lithium action in multiple behaviors (13, 34–37); deletion of Gsk3a or Gsk3b mimics lithium action in mouse behaviors (13, 20, 34); and, as shown here, lithium-sensitive behaviors (and dissociation of the β-arrestin-2 complex) were reversed by raising Gsk3 expression in the brain, demonstrating that these actions of lithium are specifically and causally related to GSK-3 inhibition.

Applying these criteria to validate GSK-3 in the therapy of BPD is more challenging, but early data are encouraging. GSK-3 N-terminal phosphorylation, a marker for GSK-3 inhibition, is increased in PBMCs from patients with BPD treated with lithium (51) and is reduced in PBMCs of untreated patients with BPD compared with that of healthy controls, implying increased baseline GSK-3 activity (33). Furthermore, olanzapine, an alternative medication for BPD, has also been reported to be a direct GSK-3 inhibitor (52). The observation that a structurally distinct GSK-3 inhibitor may have similar therapeutic actions as lithium supports the hypothesis that GSK-3 is the therapeutically relevant target of lithium in the treatment of BPD.

Other GSK-3 transgenic mouse lines have been reported, but these were not tested for reversal of lithium-sensitive behaviors (30, 53, 54). Interestingly, overexpression of the phosphorylation-defective GSK-3β<sup>PSA</sup> increases locomotor activity, which was interpreted as a manic-like state (53). Similarly, knockin mice in which N-terminal phosphorylation sites in Gsk3a and Gsk3b were mutated to alanine, preventing inhibitory phosphorylation, show hyperlocomotion in a novel environment (33, 55), enhanced locomotor response to amphetamine, increased immobility in the FST and tail suspension test, and reduced time in the open arms of the elevated plus maze (33). All of these behaviors are opposite to the behaviors observed with lithium or Gsk3b haploinsufficiency and are therefore consistent with GSK-3 as the relevant target of lithium in these behaviors. It should be pointed out, however, that Ackermann et al. observed reduced immobility in the FST and increased time in the open arms of the EPM (55); although the hyperactivity of the mice in their study may make it difficult to interpret increased swimming activity in the FST, nevertheless the reason for the differences between these 2 studies is not clear, as both groups used mice on a similar, albeit mixed, genetic background.

Many lithium-sensitive behaviors in mice require β-arrestin-2, and assembly in the striatum of a complex that includes β-arrestin-2, Akt, and PP2A is sensitive to lithium. β-Arrestin-2 recruits PP2A to dephosphorylate and inactivate Akt (Figure 4). As Akt phosphorylates and inactivates GSK-3, assembly of this complex is predicted to enhance GSK-3 activity. Lithium interferes with stability of this β-arrestin complex, and, by preventing PP2A-dependent dephosphorylation of Akt, enhances Akt-mediated inhibition of GSK-3. This model is strongly supported by the observations that either β-arrestin-2 knockout or lithium enhances GSK-3 phosphorylation in vivo. However, while lithium can disrupt the direct interaction of recombinant β-arrestin-2 and Akt, this was done in the presence of 50 mM lithium, and whether interaction of the purified proteins is sensitive to more therapeutically relevant lithium concentrations has not been reported. Alternatively, we propose that GSK-3 plays a functional role in complex stability. In support of this hypothesis, structurally diverse GSK-3 inhibitors disrupted the β-arrestin-2 complex in striatal extracts, loss of one copy of Gsk3b also disrupted the complex in vivo, and overexpression of Gsk3b restored complex formation in the presence of lithium. These data show that GSK-3 is required in vivo and in vitro for the stability of this complex (Figure 4A) and support the hypothesis that direct inhibition of GSK-3 by lithium contributes substantially to disassembly of the β-arrestin-2/Akt/PP2A complex (Figure 4B).

The IC<sub>50</sub> for lithium inhibition of GSK-3 is approximately 1 mM, at the high end of the therapeutic window for lithium therapy. However, lithium treatment enhances inhibitory phosphorylation of GSK-3, both in vivo and in cell culture, and this has been proposed to amplify the in vivo response to lithium in target tissues (31, 56). Thus, GSK-3 plays a positive role in its own activation by promoting activation of a phosphatase that removes N-terminal inhibitory phosphate groups on GSK-3 (57) and by promoting dephosphorylation and inhibition of Akt by stabilizing β-arrestin-2/Akt/PP2A interaction in the striatum. Lithium, through direct inhibition of GSK-3, blocks both mechanisms of autoactivation of GSK-3, providing at least two mechanisms to enhance lithium inhibition of GSK-3 in a tissue-dependent manner. Conversely, sensitivity
to lithium is markedly affected by the level of GSK-3, and small increases in GSK-3 expression can therefore attenuate sensitivity to lithium, as shown here and discussed previously (57).

While the behavioral effects of Gsk3β haploinsufficiency reported here and in prior work from this and other groups are highly concordant, one group reported that they were unable to observe behavioral phenotypes in Gsk3β-/- mice (58). However, these behaviors were tested in mice with a mixed genetic background that included the 129 strain, whereas the mice in this study and in the work of Beaulieu et al. were backcrossed into the C57BL/6 background (13). In this context, it should be noted that 129 mice are insensitive to lithium in amphetamine-induced hyperlocomotion, whereas C57BL/6 mice are highly sensitive (59), an intriguing observation that deserves further study, as it may help to explain the variable response to lithium in patients with BPD.

In summary, we have shown that overexpression of Gsk3β rescues the behavioral phenotypes observed in Gsk3β heterozygotes and lithium-treated mice and that direct inhibition of GSK-3 by lithium destabilizes the interaction of β-arrestin-2/Akt/PP2A in the striatum. Taken together with prior work using alternative GSK-3 inhibitors and genetic studies using both loss of function and knockin of constitutively active forms Gsk3β, these findings provide strong support for GSK-3 as the relevant target of lithium in animal behaviors. Important questions to address in future work are how GSK-3 regulates these behaviors at a molecular level and, importantly, in which neuronal populations is GSK-3 function important for regulating these behaviors.

Methods

Generation of transgenic mice. The Gsk3β transgene was generated with Xenopus Gsk3β (93% identical to mouse and human Gsk3β and 97% similar to mouse and human Gsk3β; ref. 27), provided by David Kimelman (University of Washington, Seattle, Washington, USA), and driven by the mouse prion promoter (MoPrp.Xho; gift of David Borchelt, Johns Hopkins University, Baltimore, Maryland, USA) (39, 60). A 6Xhis tag was added to the C terminus to distinguish endogenous from transgenic GSK-3β. Linearized plasmid was purified and injected into C57B6/129 heterozygous blastocysts by the University of Pennsylvania Transgenic and Chimeric Mouse Facility for generation of mice. Two founders were used to establish distinct lines, PrpGsk3βL64 and Prp-Gsk3βL64, which were backcrossed into the C57/B6 strain for more than 10 generations. Both transgenic lines had normal litter sizes, and the transgene was transmitted in normal Mendelian fashion. Tissue from 3-month-old mice of each line was harvested for molecular analysis. Several brain regions were microdissected, and transgene expression was confirmed for both lines by RT-PCR. Transgenic protein expression was assessed by Western blot for the constitutively active forms Gsk3β, these findings provide strong support for GSK-3 as the relevant target of lithium in animal behaviors.

Behavioral testing. In regard to strain background, behavioral analysis was initially performed in mice backcrossed a minimum of 5 to 10 generations into C57/B6; all behavioral data were confirmed in a larger cohort backcrossed more than 10 generations into C57/B6. Porsolt FST, hole board exploratory behavior, and EZM were performed, as described previously (20), on days 7, 9, and 11 of lithium treatment. The order of the respective tests was varied to avoid an order effect.

Lithium dosing and testing schedule. Control and transgenic mice were allowed free access to water and standard mouse chow throughout the experiment. For lithium treatment, mice were fed 0.2% (w/w) LiCl mouse chow (Harlan Teklad) ad lib for 3 days, followed by 0.4% (w/w) LiCl mouse chow for the duration of the experiment (20). All mice had access to supplemental 450 mM NaCl drinking solution.

Immunoblotting and immunoprecipitations. Frontal cortex, hippocampus, hypothalamus, striatum, nucleus accumbens, amygdala, olfactory bulb, and cerebellum were harvested on day 12 of lithium treatment, frozen in liquid nitrogen, and stored at −80°C. All behavior experiments and tissue harvest took place between 8:00 AM and 12:30 PM. Antibodies to glycogen synthase, phosphorylated glycogen synthase (GSβS641P), and Akt were from Cell Signaling Technology. Anti-β-arrestin-2 antibodies were from Cell Signaling Technology. Anti-β-tubulin antibodies were from Promega. Frozen samples of dissected brain regions were homogenized with a Polytro ProT10-35 Tissue Homogenizer in 200 μl 0.75% NP-40 lysis buffer (61). Protein content was determined by Bradford assay. Samples from individual animals (5–10 μg per lane) were analyzed by SDS-PAGE and immunoblotting. Immunoblots were visualized by ECL or ECL plus (Amer sham). To measure band intensity in immunoblots, filters were probed with infrared-labeled secondary antibodies (Rockland Inc.), imaged with a LI-COR Odyssey Infrared Imager according to the manufacturer’s instructions, and quantitated using LI-COR software (LI-COR Biosciences). For immunoprecipitations, striatal lysates were prepared as above and pooled from 3 mice per group. One mg of striatal lysate was used for immunoprecipitation with an antibody to Akt immobilized on sepharose beads (Cell Signaling Technology). Immunoprecipitation was conducted overnight at 4°C, followed by immunoblotting with an anti-PP2A antibody (Cell Signaling Technology).

Statistics. A 1-tailed Student’s t test was performed on all behaviors to test for gender differences. No significant gender differences were found so the data were combined. For all other data, a 1-way ANOVA was used, followed by Dunn’s post-hoc analysis when a significant difference was found among groups. The level of significance was set at P < 0.05. Histograms show mean values ± SEM throughout.

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Address correspondence to: Peter S. Klein, 364 Clinical Research Building, 415 Curie Blvd., Philadelphia, Pennsylvania 19104, USA. Phone: 215.898.2179; Fax: 215.573.4320; E-mail: pklein@mail.med.upenn.edu.