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Synapse-specific IL-1 receptor subunit reconfiguration augments vulnerability to IL-1β in the aged hippocampus

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In the aged brain, synaptic plasticity and memory show increased vulnerability to impairment by the inflammatory cytokine interleukin 1β (IL-1β). In this study, we evaluated the possibility that synapses may directly undergo maladaptive changes with age that augment sensitivity to IL-1β impairment. In hippocampal neuronal cultures, IL-1β increased the expression of the IL-1 receptor type 1 and the accessory coreceptor AcP (proinflammatory), but not of the AcPb (prosurvival) subunit, a reconfiguration that potentiates the responsiveness of neurons to IL-1β. To evaluate whether synapses develop a similar heightened sensitivity to IL-1β with age, we used an assay to track long-term potentiation (LTP) in synaptosomes. We found that IL-1β impairs LTP directly at the synapse and that sensitivity to IL-1β is augmented in aged hippocampal synapses. The increased synaptic sensitivity to IL-1β was due to IL-1 receptor subunit reconfiguration, characterized by a shift in the AcP/AcPb ratio, paralleling our culture data. We suggest that the age-related increase in brain IL-1β levels drives a shift in IL-1 receptor configuration, thus heightening the sensitivity to IL-1β. Accordingly, selective blocking of AcP-dependent signaling with Toll–IL-1 receptor domain peptidomimetics prevented IL-1β–mediated LTP suppression and blocked the memory impairment induced in aged mice by peripheral immune challenge (bacterial lipopolysaccharide). Overall, this study demonstrates that increased AcP signaling, specifically at the synapse, underlies the augmented vulnerability to cognitive impairment by IL-1β that occurs with age.

AcP | AcPb | neuroinflammation | receptor sensitivity | LTP

Interleukin 1β (IL-1β) is a key proinflammatory cytokine associated with age-related cognitive decline (1–3). A growing body of evidence indicates that synaptic plasticity (4), learning, and memory (5) are more vulnerable to impairment by IL-1β with age. After systemic immune activation (e.g., *Escherichia coli* or trauma), aged, but not young, rodents show deficits selective for hippocampal-dependent memory (6–11), long-term potentiation (LTP) (12, 13), and brain-derived neurotrophic factor (BDNF) signaling (14), all of which are blocked by brain infusion of the IL-1 receptor antagonist (IL-1ra) (6, 7, 12, 14). Although investigation of underlying mechanisms has largely focused on inflammatory responses of glia (15), it is possible that synapses themselves may also undergo maladaptive changes with age that augment vulnerability to inflammation. We explored the hypothesis that the suppression of BDNF signaling, LTP, and memory may be driven by an increased sensitivity to IL-1β that occurs directly at synapses.

Canonical IL-1β signaling promotes inflammation through a heterodimeric receptor comprising the ligand-binding subunit, IL-1 receptor type 1 (IL-1R1), and the accessory protein subunit (AcP), which functions as an essential coreceptor (16). IL-1β binding to IL-1R1 is followed by AcP recruitment (17), and the ensuing juxtaposition of the Toll/IL-1 receptor (TIR) domains of IL-1R1 and AcP engages the adapter protein MyD88 (18), a fundamental step for activation of downstream effectors of inflammation (e.g., stress kinase p38) (19). In the CNS, however, IL-1β signaling can be additionally mediated by a second accessory protein, AcPb—an alternative splice variant of AcP (20) that is expressed only in neurons (21, 22). Although AcP and AcPb have identical extracellular segments, the intracellular C-terminal tail is extended in AcPb, potentially affecting the TIR domain structure (21). Indeed, although both AcP and AcPb physically interact with IL-1R1 in response to IL-1β, only AcP recruits MyD88, such that AcPb is unable to activate canonical downstream effectors of IL-1β proinflammatory signaling (20, 21). As would be predicted, CNS induction of inflammatory cytokines in response to bacterial lipopolysaccharide (LPS) is absent in AcP-deficient mice, but intact in AcPb-deficient mice (21), demonstrating that only AcP activates the proinflammatory response. Consistent with the emerging data that AcP and AcPb serve different functions, studies using AcP- and AcPb-knockout mice demonstrate that AcP mediates inflammation and neuronal damage in a model of multiple sclerosis, whereas AcPb serves a protective role, promoting neuronal survival after the induction of acute inflammation (21) and excitotoxicity (23) (Fig. 1*A*).

Here, we demonstrate that the balance between AcP and AcPb levels in hippocampal neurons is a fundamental determinant of the downstream consequences of IL-1β exposure. Using an assay that we developed to track LTP directly in synaptosomes, we demonstrate

Significance

There is a growing understanding that inflammation impairs synaptic plasticity and cognition and that the aged brain has an elevated sensitivity to cognitive impairment by the proinflammatory cytokine interleukin 1β (IL-1β). IL-1β activates different pathways via AcP (proinflammatory) or AcPb (prosurvival) IL-1 receptor subunits. This study demonstrates that the IL-1 receptor subunit system undergoes an age-dependent reconfiguration in hippocampal synapses. This previously undescribed reconfiguration, characterized by an increase in the AcP/AcPb ratio, is responsible for potentiating impairments of synaptic plasticity and memory by IL-1β. Our data reveal a previously unidentified mechanism that explains the age-related vulnerability of hippocampal function to impairment by inflammation and adds another dimension beyond glia to understanding how inflammation causes cognitive decline in aging.

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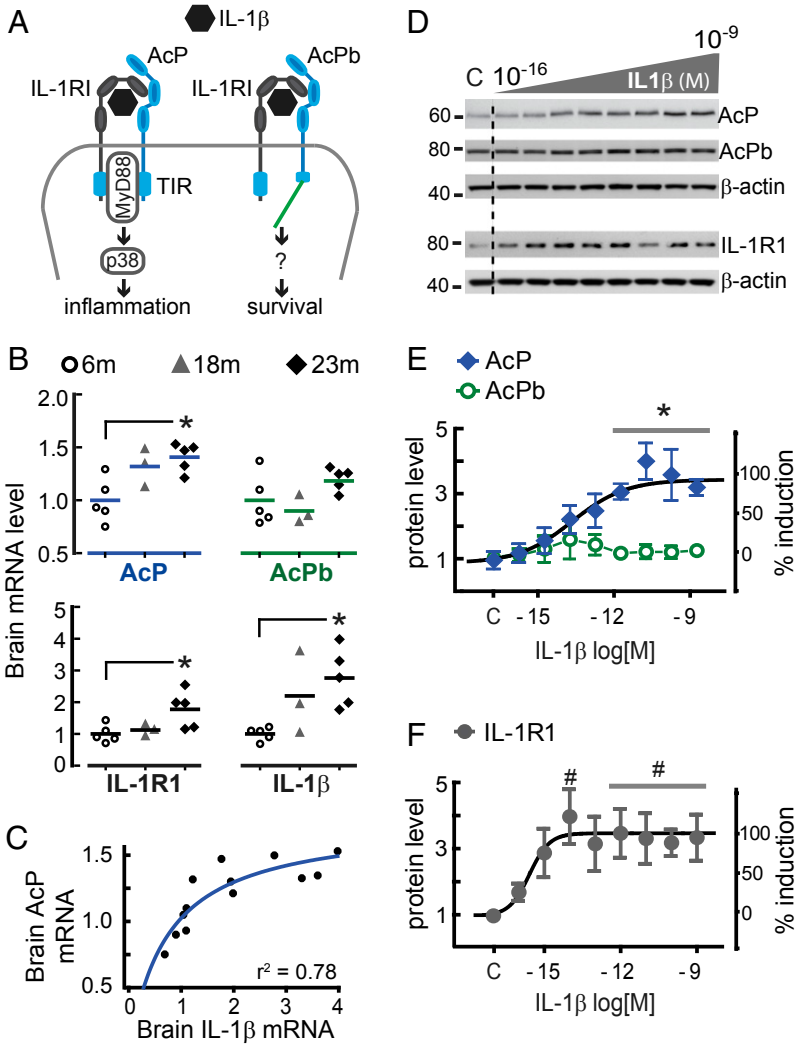

Fig. 1. Aging and IL-1β reconfigure the IL-1 receptor system. (*A*) Neuronal IL-1 receptor system. Note that AcPb has a long C-terminal region potentially blocking MyD88 recruitment (see text). (*B*) Whole-brain mRNA expression of AcP, AcPb, IL-1R1, and IL-1β in mice at 6 ($n = 5$), 18 ($n = 3$), and 23 ($n = 5$) mo old, determined by qPCR. Levels were normalized with values from 6-mo-old mice and are presented in dot plots (mean indicated by a bar). GAPDH level was used as internal control. *$P < 0.05$ (Kruskal–Wallis, Dunn's post hoc test). (*C*) Correlation between AcP and IL-1β relative expression in the whole set of brain samples ($r^2 = 0.78$; $n = 13$). (*D*) Western blot analysis of AcP, AcPb, and IL-1R1 in primary rat hippocampal neurons (5–7 DIV) treated with IL-1β (3 h, 0.3 fM to 3 nM, in 10-fold increments). β-actin was used as loading control. (*E* and *F*) AcP, AcPb (*E*; $n = 5$), and IL-1R1 (*F*; $n = 4$) densitometry values were normalized to vehicle-treated cells (c, control); results are from five independent experiments (neurons from different embryo litters). *AcP* vs. *AcPb*, $P < 0.0001$ (two-way ANOVA main effect of receptor, $F_{1,54} = 46.1$); *$P < 0.01$ (Bonferroni post hoc test). *IL-1R1* vs. *AcPb*, $P < 0.0001$ (two-way ANOVA main effect of receptor, $F_{1,51} = 46.8$); #$P < 0.05$ (Bonferroni post hoc test). Concentration–response relationships were fitted to the Hill equation (black traces), and the highest induction of AcP (*E*) and IL-1R1 (*F*) was set as the E_{max} (maximal effect, 100%). Data are presented as mean ± SEM.

that IL-1β impairs LTP directly at the synapse. Synaptic sensitivity to IL-1β is augmented in the aged hippocampus and is due to an increased AcP relative to AcPb expression, which biases signaling toward AcP-dependent (proinflammatory) signaling. We extend our findings in vivo to specifically investigate the role of AcP-dependent signaling in the memory impairments induced by peripheral immune challenge in aged mice.

Results

IL-1β Induces AcP, but Not AcPb, Expression in Hippocampal Neurons. We first explored whether chronic IL-1β signaling in the aged brain was associated with altered expression of IL-1 receptor subunits, because it is known that IL-1β amplifies its own inflammatory responses by inducing gene expression of IL-1 system components (19), including IL-1β (24) and IL-1R1 (25, 26). Analysis of whole brain homogenates from 6-, 18-, and 23-mo-old mice revealed an age-dependent up-regulation of AcP, IL-1R1, and IL-1β mRNA, but not AcPb mRNA (Fig. 1*B*). The rise in IL-1β gene expression across age positively correlated with AcP ($r^2 = 0.78$; $n = 13$; Fig. 1*C*), but not with AcPb mRNA levels ($r^2 = 0.29$), suggesting that age-related elevations of IL-1β selectively increase AcP, but not AcPb. To directly test whether IL-1β modulates neuronal expression of IL-1 receptor components, we evaluated AcP, AcPb, and IL-1R1 protein levels after IL-1β treatment in cultured rat hippocampal neurons, an experimental system devoid of nonneuronal brain cells, notably microglia and astrocytes, which are responsive to IL-1β. Cultured hippocampal neurons (5–7 d in vitro; DIV) were treated with 3 nM (50 ng/mL) IL-1β and harvested at different time points (0, 1, 3, 6, 12, and 24 h). AcP (~62 kDa) and AcPb (~80 kDa) were identified by Western blot by their molecular masses (Fig. S1). No significant change in AcPb protein expression was found at any time point after IL-1β treatment. However, after 3 h of IL-1β treatment, there was a significant increase in AcP ($P < 0.05$; Fig. S1). Additional experiments

demonstrated that IL-1β treatment (3 h) across a range of concentrations (0.3 fM to 3 nM) increased AcP protein expression in a concentration-dependent manner (IC_{50}: 17 fM; $r^2 = 0.97$), whereas AcPb levels were not significantly changed at any concentration tested (Fig. 1 *D* and *E*). In parallel with the effect on AcP levels, IL-1β treatment (3 h) increased IL-1R1 protein levels in a dose-dependent manner (IC_{50}: 0.26 fM; $r^2 = 0.94$; Fig. 1 *D* and *F*). Overall, these data indicate that IL-1β reconfigures the IL-1 receptor system by increasing IL-1R1 and AcP (but not AcPb) protein levels in hippocampal neurons, a series of findings confirmed by mRNA quantification using quantitative RT-PCR (RT-qPCR) (Fig. S1). These results suggest that IL-1β exposure may bias IL-1β signaling toward IL-1R1–AcP proinflammatory responses.

Increased AcP/AcPb Ratio Sensitizes the IL-1β Response in Hippocampal Neurons. We next hypothesized that IL-1R1 and AcP up-regulation by IL-1β may sensitize hippocampal neuronal responses to subsequent IL-1β challenge. We tested this idea using BDNF signaling as an endpoint, based on our previous work demonstrating that IL-1β suppresses BDNF signaling in hippocampal neuronal cultures (27, 28).

We have consistently found that 3 nM IL-1β significantly impairs neuronal BDNF signaling (27–29). Fig. S2 shows that BDNF signaling in primary rat hippocampal neurons (5–7 DIV) is also reduced by 30 and 300 pM IL-1β, but not by 0.3 or 3 pM IL-1β. Thus, to test whether treatment with IL-1β sensitizes neuronal responses to subsequent IL-1β exposure, cultured rat hippocampal neurons were preincubated with IL-1β (3 nM, 3 h), washed, and immediately treated with BDNF (50 ng/mL) in the presence of 3 pM IL-1β (hereafter referred to as IL-$1\beta_{low}$) (Fig. 2*A*). In control neurons not exposed to IL-1β (neither pretreatment nor cotreatment), BDNF induced the phosphorylation of its receptor, TrkB, as well as phosphorylation of the downstream targets Akt and CREB (Fig. 2 *B*–*E*). Consistent with our previous findings that BDNF signaling is only suppressed in the presence of IL-1β (27–29), BDNF signaling was not affected by IL-1β pretreatment alone. Similarly, BDNF signaling was not affected by IL-$1\beta_{low}$ exposure in the absence of IL-1β pretreatment. In contrast, after IL-1β pretreatment, BDNF signaling was significantly impaired by IL-$1\beta_{low}$ (Fig. 2 *B*–*E*), supporting the idea that IL-1β exposure sensitizes neurons to subsequent IL-1β challenge, evoking responses at levels of IL-1β that are normally ineffective for impairing BDNF signaling. To directly test the possibility that IL-1β can potentiate its own signaling, we examined the stress kinase p38, a downstream effector of the AcP-dependent IL-1β signaling (21). We found that IL-$1\beta_{low}$ activates p38 in neurons pretreated with 3nM IL-1β (3 h), whereas no changes were detected in the activation levels of Src, a downstream kinase recently associated with AcPb-dependent IL-1β signaling (22) (Fig. 2 *F* and *G*). These findings further support the idea that IL-1β exposure biases IL-1β signaling toward AcP proinflammatory responses.

Because 3 h treatment with IL-$1\beta_{low}$ (3 pM) enhanced IL-1R1 and AcP expression (Fig. 1 *D*–*F*), we hypothesized that even IL-$1\beta_{low}$ may increase sensitivity to IL-1β. As predicted, we found that pretreatment with IL-$1\beta_{low}$ (3 h) is sufficient to sensitize the IL-1β response to subsequent IL-$1\beta_{low}$ challenge (Fig. S3), thus supporting a causal link between the IL-1R–AcP up-regulation and the enhanced inflammatory potency of IL-1β to suppress BDNF signaling in hippocampal neurons. Further, to confirm a role of IL-1R1 in the sensitization of the IL-1β response, the impairment of BDNF signaling by IL-$1\beta_{low}$ was blocked by IL-1ra, a cytokine that blocks the binding site for IL-1β on IL-R1 (16) (Fig. S3). Although BDNF activates multiple signaling pathways (PI3K/Akt, MAPK/ERK, and PLC/CaMK) (30), we found that the sensitized IL-1β response suppressed BDNF-induced activation of Akt, but not phosphorylation of CREB (Fig. S3 and Fig. 2 *B*, *D*, and *E*).

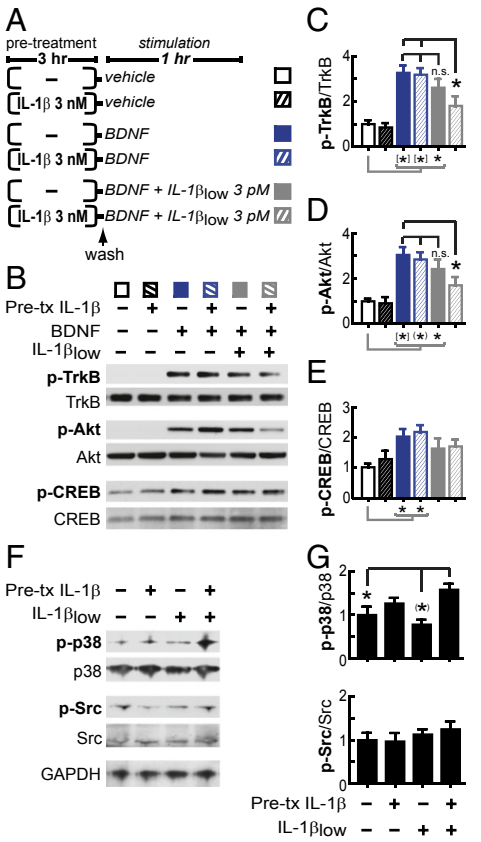

Fig. 2. IL-1β pretreatment sensitizes the inflammatory branch of IL-1β signaling in rat hippocampal neurons. (*A*) Experimental design: Primary rat hippocampal neurons (5–7 DIV) were preincubated with 3 nM IL-1β or vehicle for 3 h, washed, and stimulated with BDNF with or without IL-$1\beta_{low}$ for 1 h, as indicated. (*B*) Western blot analysis of phosphorylated and total levels of TrkB, Akt, and CREB after the above treatments. (*C*–*E*) Relative levels of p-TrkB (Tyr-490)/TrkB (*C*), p-Akt (Ser-473)/Akt (*D*), and p-CREB (Ser-133)/CREB (*E*). Data were normalized with vehicle-treated neurons (control) ($n = 9$; nine independent experiments). $^{*}P < 0.05$; $^{(*)}P < 0.01$; $^{[*]}P < 0.001$; n.s., not significant (ANOVA, Tukey's post hoc test). (*F* and *G*) Primary rat hippocampal neurons (5–7 DIV) were preincubated with 3 nM IL-1β or vehicle for 3 h, washed, and stimulated with IL-$1\beta_{low}$ for 20 min, as indicated. Western blot analysis of phosphorylated and total levels of p38 and Src followed the above treatments. (*G*) Relative levels of p-p38 (Thr-180/Tyr-182)/p38 and p-Src (Tyr-416)/Src. Data were normalized with vehicle-treated neurons (control) ($n = 8$; eight independent experiments). $^{*}P < 0.05$; $^{(*)}P < 0.01$ (ANOVA, Tukey's post hoc test). Data are presented as mean ± SEM.

To determine whether the increased AcP relative to AcPb expression is responsible for the potentiated inflammatory response to IL-1β in neurons that had been preincubated in IL-1β, we investigated whether the impairment of BDNF signaling by IL-1β can be blocked by reducing the AcP/AcPb ratio, either by inhibiting AcP expression or by increasing AcPb levels. First, we reduced the AcP/AcPb ratio by using short-hairpin RNA (shRNA) to specifically knock down AcP expression, without affecting AcPb levels ($P = 0.04$; Fig. 3 *A*–*D*). As predicted, compared with control-shRNA transfection, AcP knockdown reduced the IL-1β impairment of BDNF-dependent Akt activation in primary rat hippocampal neurons preincubated with 3 nM IL-1β (3 h) and challenged with IL-$1\beta_{low}$ (Fig. 3 *E* and *F*). Similarly, in AcPb-overexpressing neurons, BDNF induced a significant increase in p-Akt levels, despite IL-1β pretreatment and subsequent IL-$1\beta_{low}$ challenge (Fig. 4 *A*–*C*). Overall, these results indicate that the IL-1β inflammatory response in hippocampal neurons depends on AcP and can be attenuated by AcPb, thus supporting the idea that the AcP/AcPb ratio modulates downstream effects of IL-1β signaling.

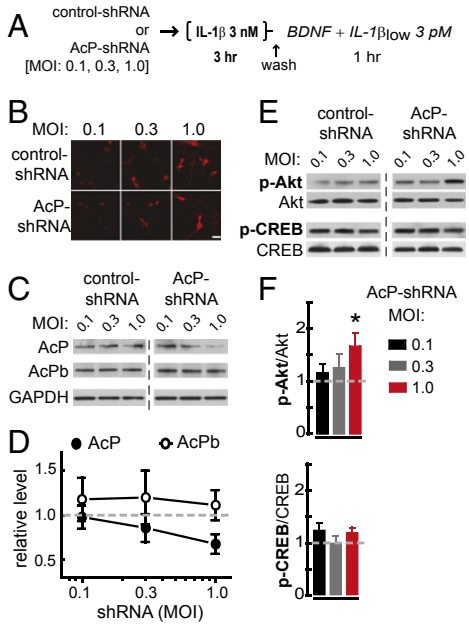

Fig. 3. AcP knockdown attenuates the neuronal IL-1β inflammatory response. (*A*) Experimental design. Both control– and AcP–shRNA-transfected neurons (at 3 DIV) were treated identically (at 6–7 DIV): All cultures were preincubated with 3 nM IL-1β for 3 h, washed, and stimulated with BDNF (50 ng/mL) with IL-1β_{low} (3 pM) for 1 h, as indicated. AcP–shRNA targets a sequence in the unique 3′ terminal segment of the rat AcP mRNA, a sequence absent in rat AcPb mRNA (*SI Methods*). (*B*) RFP detection in primary rat hippocampal neurons transfected with increasing MOI of lentivirus. (Scale bar: 50 μm.) (*C*) Western blot analysis of AcP and AcPb levels; GAPDH was used as loading control. (*D*) Quantification: AcP ($n = 8$) and AcPb ($n = 8$) levels in AcP–shRNA-transfected neurons were normalized with corresponding levels from neurons transfected with equivalent MOI of control shRNA (dashed line). Results are from five independent experiments (neurons from different embryo litters). *AcP* vs. *AcPb* levels, $P = 0.04$ (two-way ANOVA main effect of shRNA; $F_{3,44} = 2.99$). (*E*) Western blot analysis of p-Akt (Ser-473) and p-CREB (Ser-133) levels (Akt and CREB levels as loading controls). (*F*) Quantification: After densitometry analysis, p-Akt ($n = 8$) and p-CREB ($n = 7$) levels in AcP–shRNA-transfected neurons were normalized with corresponding levels from neurons transfected with equivalent MOI of control shRNA (dashed line); results are from five independent experiments. *AcP–shRNA* vs. *control-shRNA* (two way ANOVA): p-Akt, $P = 0.01$, effect of shRNA, $F_{1,41} = 7.07$; p-CREB, $P = 0.17$, effect of shRNA concentration, $F_{1,36} = 1.96$. $^{*}P < 0.05$ (Bonferroni post hoc test for p-Akt). Data are presented as mean ± SEM.

Because both AcP and AcPb are recruited to IL-1R1 in response to IL-1β (20, 21), AcPb may attenuate AcP-dependent responses by competing for IL-1R1 binding, thus attenuating IL-1β–induced suppression of BDNF signaling (Fig. 2). Confirming that AcP is recruited to the IL-1R1–IL-1β complex (17), AcP coimmunoprecipitated with IL-1R1 after IL-1β treatment of cultured rat hippocampal neurons (Fig. 4*D*). To test whether AcPb competes with AcP for IL-1R1 binding, IL-1R1 was immunoprecipitated after IL-1β treatment of AcPb- and control-transfected neurons. Compared with controls, IL-1R1–AcP coimmunoprecipitation was reduced in neurons transfected with AcPb (Fig. 4*E*). This result strongly suggests that AcP and AcPb compete for the ligand-binding chain of the IL-1 receptor, consistent with our hypothesis that the relative abundance of AcP/AcPb modulates the extent to which IL-1β triggers proinflammatory cascades.

Our in vitro data indicate that up-regulation of AcP and IL-1R1, which occurs in the hippocampus during the course of aging (Fig. 1*B*), potentiates the IL-1β inflammatory response in rat hippocampal neurons. Because AcP and IL-1R1 have been detected in postsynaptic density (PSD) fractions from adult rat hippocampus (31), we hypothesized that both the reconfiguration and sensitization of the IL-1 receptor may occur directly at synapses in the aged hippocampus.

Age-Dependent Increase in the Level of AcP Relative to AcPb in Hippocampal Synapses. To investigate whether age-related changes in IL-1 receptor components occur specifically at the synapse, we quantified AcP, AcPb, and IL-1R1 protein levels in hippocampal synaptosomes [presynaptic terminals attached to postsynaptic

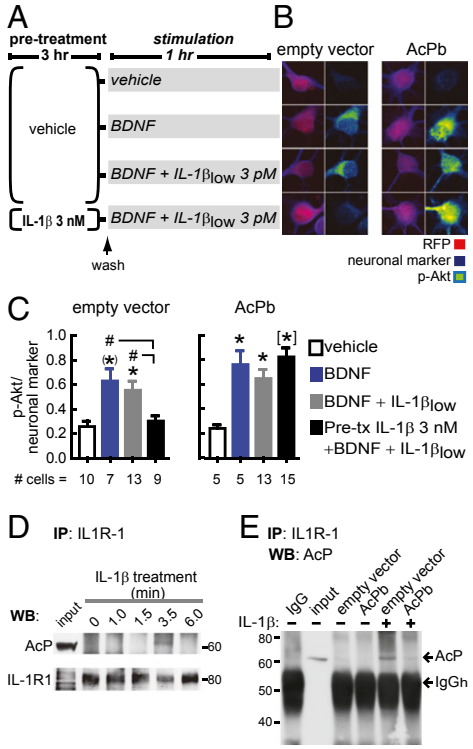

Fig. 4. AcPb attenuates the neuronal IL-1β inflammatory response. (*A*) Primary rat hippocampal neurons were transfected with empty or AcPb-containing vectors at 3 DIV (*AcPb* gene sequence under the CMV promoter). After 3–4 d, neurons were preincubated with 3 nM IL-1β or vehicle for 3 h, washed, and stimulated with BDNF with or without IL-1β_{low} for 1 h, as indicated. (*B*) Phosphorylated levels of Akt (p-Akt; Ser-473) were assessed by immunofluorescence in RFP (reporter gene)-positive cells treated as indicated in *A*. Pan neuronal marker staining was used for cell-volume normalization. Representative images are shown. (Scale bar: 10 μm.) (*C*) p-Akt/pan-neuronal marker levels following the above treatments. The number of analyzed neurons is shown at the bottom of each treatment. Transfection itself did not interfere with BDNF signaling. Akt activation by BDNF was not impaired by IL-1β_{low} treatment alone (i.e., in the absence of IL-1β pretreatment) in either control- or AcPb-transfected neurons. Consistent with experiments in nontransfected cells (Fig. 2*D*), BDNF induction of p-Akt was prevented by IL-1β_{low} challenge after IL-1β pretreatment in control-transfected neurons. Bar graphs show data from a representative experiment (one out of four independent experiments). $^{*}P < 0.05$; $^{(*)}P < 0.01$; $^{[*]}P < 0.001$ vs. control; $^{\#}P < 0.05$ (ANOVA, Tukey's post hoc test). (*D*) Neurons were treated with 3 nM IL-1β for the indicated times. IL-1R1 immunoprecipitation (IP) was followed by Western blot (WB) for AcP and IL-1R1 ($n = 3$; three independent experiments). IL-1R1–AcP interaction was detected after 3.5 min, but not after 6 min, of IL-1β treatment, possibly due to MyD88 binding to the C-terminal domain of IL-1R1 (18), the same region recognized by the antibody used for IL-1R1 IP. (*E*) IL-1R1 IP was performed in neurons transfected with empty or AcPb-containing vectors. Neurons were treated with vehicle or IL-1β (3 nM, 3.5 min), as indicated. Membrane was probed for AcP; shown is a representative experiment ($n = 3$; three independent experiments). Data are presented as mean ± SEM.

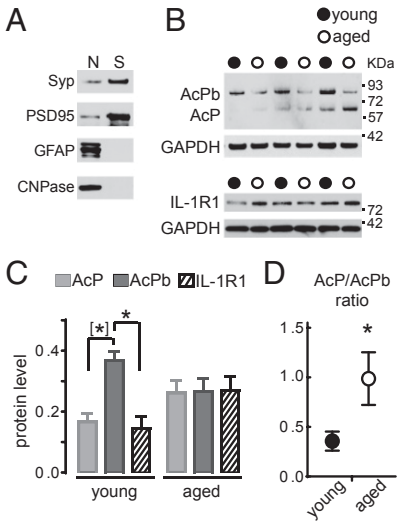

Fig. 5. Aging reconfigures the IL-1 receptor in mouse hippocampal synaptosomes. (*A*) Hippocampal synaptosomes from young and middle-aged mice were purified by density gradient. Purity was evaluated by Western blot analysis of the synaptic markers synaptophysin (Syp) and PSD95 in nuclear (N) and synaptosome (S) fractions. Expression of astrocyte (glial fibrillary acidic protein; GFAP) and oligodendrocyte (2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNPase) markers was not detected in synaptosome fractions. (*B*) AcP, AcPb, and IL-1R1 proteins detected by Western blot in hippocampal synaptosome samples. (*C*) Quantification of AcP, AcPb (n = 11 samples/group), and IL-1R1 (n = 7 samples per group) protein levels in synaptosomes from young (7–8 mo) and middle-aged (13–15 mo) mice. Fresh hippocampi from two mice were pooled for each sample. Values were normalized with corresponding GAPDH levels. AcP and AcPb detection for each sample was performed in duplicate, and values were averaged before statistical analysis. $*P < 0.01$; $^{[*]}P < 0.001$ vs. AcPb in young mice (ANOVA, Tukey's post hoc test). (*D*) AcP/AcPb protein ratio. $*P < 0.05$ (Mann–Whitney test). Data are presented as mean ± SEM.

dendritic spines (32)] from young (6–7 mo) and middle-aged (13–15 mo) mice. We verified the enrichment of synaptic proteins in our synaptosomal preparation (Fig. 5*A*). Data analysis revealed a striking age-dependent change in the expression pattern of IL-1 receptor subunits. Notably, in synaptosomes from young mice, AcPb was the most abundant subunit and was present at significantly higher levels than either AcP ($P < 0.001$) or IL-1R1 ($P < 0.01$) (Fig. 5 *B* and *C*), whereas in synaptosomes from middle-aged mice, the three subunits were expressed at similar levels (Fig. 5 *B* and *C*). Thus, although both AcP and AcPb levels in hippocampal synaptosomes changed with aging, the change was in opposite directions. As a result, the AcP/AcPb ratio shifted with age, with aging associated with a nearly threefold increased AcP/AcPb ratio in hippocampal synapses ($P = 0.033$; Fig. 5*D*).

Building on our synaptosomal data demonstrating that the AcP/AcPb ratio increases specifically in synapses with age, along with our in vitro data that AcP and IL-1R1 up-regulation by IL-1β sensitizes hippocampal neurons to subsequent low-level IL-1β challenge and impairs BDNF signaling (Figs. 1 and 2), we next investigated whether aging is accompanied by increased IL-1β sensitivity directly at synapses and whether the increased IL-1β sensitivity compromises synaptic plasticity.

Aging and IL-1β Interact to Suppress LTP in Synapses via IL-1R–AcP. We assessed the effect of IL-1β on synaptic plasticity in hippocampal synaptosomes from young (6–7 mo) and middle-aged (13–15 mo) mice using a novel approach that we have developed to track LTP in freshly isolated synaptosomes. Our method induces LTP in synaptosomes by chemical stimulation (chemical LTP; cLTP) (33–39) and tracks the insertion of glutamate AMPA receptors (GluR1) into the postsynaptic surface, the first critical step for potentiation of synaptic transmission (40). Using flow cytometry, we quantify GluR1 surface expression in synaptosomal preparations at the single-synaptosome level. We refer to this approach as "Fluorescence Analysis of Single-Synapse Long-Term Potentiation" (FASS-LTP). Our method first identifies synaptosomes by size using calibrated beads (Fig. 6 *A* and *B*), as described (41). Consistent with the average size of synaptosomes (32), the subset of particles between 0.5 and 3.0 μm in P2 synaptosomal fractions (42) is highly enriched in synaptosomes, as demonstrated in our preparation by the high proportion of size-gated particles that coexpress synaptophysin and PSD95 (> 60%) and synapsin-I and PSD95 (> 70%) (Fig. 6*C*). Next, to identify potentiated synapses, our method uses antibodies specific for extracellular epitopes on GluR1 and neurexin-1β (Nrx1β) (Fig. 6*D*), a presynaptic adhesion molecule stabilized at the membrane surface by synaptic activity (43). $GluR1^+Nrx1\beta^+$ double-labeling ensures that we are analyzing intact synaptosomes that contain both presynaptic and postsynaptic elements. Fluorescence analysis of the size-gated population after cLTP induction (45 min, glycine–KCl stimulation; Methods) identifies an increased proportion of $GluR1^+Nrx1\beta^+$ double-positive events over basal levels (Fig. 6*D*), demonstrating that FASS-LTP detects activity-dependent plasticity in synaptosomes. Importantly, the cLTP response in isolated synaptosomes is sustained (159.8 ± 23.0% $GluR1^+Nrx1\beta^+$ events over basal after 75 min; $P = 0.04$; $n = 6$; Mann–Whitney test) and depends on NMDA receptor activation, because it is blocked by the NMDA receptor antagonist AP5 [50 μM] ($P = 0.02$; $n = 5$; Mann–Whitney test).

Using FASS-LTP, we tested whether chronic IL-1β signaling in aging (Fig. 1*B* and ref. 3) sensitizes the IL-1β response in hippocampal synapses. Synaptosomal fractions isolated from hippocampi of young (6–7 mo) and middle-aged (13–15 mo) mice were treated with or without $IL\text{-}1\beta_{low}$ before inducing cLTP. FASS-LTP analysis revealed that, in the absence of $IL\text{-}1\beta_{low}$ treatment, the proportion of $GluR1^+Nrx1\beta^+$ events in cLTP-stimulated samples was significantly increased over basal levels in synaptosomes from both young (185 ± 22%; $P < 0.05$) and middle-aged (156 ± 11%; $P < 0.05$) mice (Fig. 6 *E* and *F*). A FASS-LTP time course (0, 15, 25, and 45 min) showed that cLTP responses in synaptosomes from middle-aged mice are significantly reduced relative to young animals ($P = 0.01$; two-way ANOVA effect of age; $F_{1,45} = 6.4$), a finding consistent with the age-dependent reduction of "conventional" electrically induced LTP in middle-aged mice (44, 45) and rats (46). Interestingly, treatment with $IL\text{-}1\beta_{low}$ did not significantly affect cLTP-induced changes in synaptosomes isolated from young mice, but totally suppressed the cLTP response in synaptosomes from middle-aged mice ($P < 0.05$; Fig. 6 *E* and *F*). These data, to our knowledge, demonstrate for the first time that the IL-1β response is sensitized in synapses from the aged hippocampus. Importantly, because glial cells are absent in the synaptosomal preparation, our finding strongly suggests that IL-1β impairs LTP by direct actions on neuronal synapses and does not require an intermediary response from IL-1β–activated glia.

Based on our data that the IL-1β inflammatory response in hippocampal neurons depends on AcP (Fig. 3), and because the IL-1R1–AcP (but not IL-1R1–AcPb) receptor recruits MyD88 via TIR domains (18, 20, 21) [canonical IL-1β signaling (19)], we hypothesized that the potentiated IL-1β suppression of cLTP in aged synaptosomes is TIR-domain–dependent. To block TIR domain signaling, we used TIR mimetics, synthetic compounds that mimic the BB-loop of the TIR domain and specifically block TIR-domain–dependent IL-1β signaling in multiple cell types (47, 48) including neurons (49). We found that the TIR mimetics AS1 (47) and EM163 (48) specifically block the sensitized signaling through

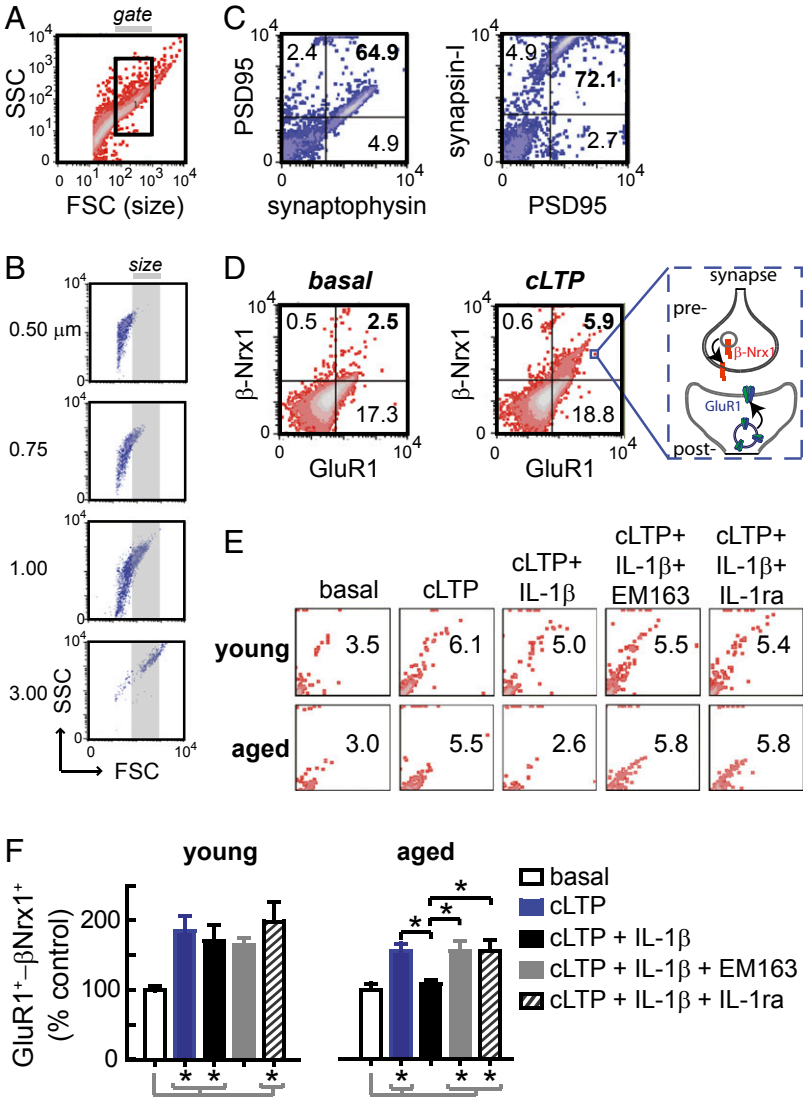

Fig. 6. Sensitized IL-1β response in aged synapses suppresses cLTP via IL-1R1–AcP signaling. (*A*) Forward scatter (FSC) vs. side scatter (SSC) dot plot showing the size–complexity profile of gated particles (inside rectangle, size-gated synaptosomes). (*B*) FSC–SSC dot plots using 0.5-, 0.75-, 1.0-, and 3.0-μm calibrated beads. Gray area represents the size range used to select putative synaptosomes particles: 0.5 μm < gated particles ≤ 3.0 μm. (*C*) Two-color parameter density plots showing synaptophysin–PSD95 (*Left*) and synapsin-I-PSD95 (*Right*) double-labeling in size-gated synaptosomes. Thresholds for endogenous/nonspecific fluorescence for each marker are set by using secondary antibody staining only (lower left quadrant). Note that a high proportion of particles coexpress presynaptic and postsynaptic markers (>60%). Representative plots of one experiment out of five are shown. (*D*, *Left* and *Center*) Two-color parameter density plots showing Nrx1β and GluR1 surface detection in size-gated synaptosomes before and after cLTP. GluR1–Nrx1β double-positive events (upper right quadrant) increase after cLTP. (*D*, *Right*) The model illustrates the insertion of Nrx1β- and GluR1-containing endosomes after cLTP detected in single events by FASS-LTP. (*E*) FASS-LTP was performed in fresh synaptosome fractions obtained from young (6–7 mo; $n = 7$) and middle-aged (13–15 mo; $n = 6$) mice. IL-1β_{low} or external solution was added 5 min before cLTP induction. EM-163 (20 μM), IL-1ra (3 μM), or equivalent volumes of external solution were added 10 min before IL-1β_{low} (3 pM) treatment. The upper right quadrant shows the proportion of GluR1–Nrx1β double-positive events detected after 45 min of cLTP in each experimental condition. (*F*) Overall data presented as mean ± SEM. *$P < 0.05$ (ANOVA, Tukey's post hoc test).

the IL-1R1–AcP complex and rescued BDNF signaling in hippocampal cultures exposed to our experimental paradigm of IL-1β sensitization (Fig. S4), thus demonstrating main roles of IL-1R1 and AcP in the sensitization of the IL-1β response. Using FASS-LTP, we then tested whether the TIR mimetic EM163 or IL-1ra can block the suppression of cLTP induced by IL-1β_{low} in aged synaptosomes. Consistent with the rescue of BDNF signaling by TIR mimetics and IL-1ra in hippocampal cultures (Figs. S3 and S4), the cLTP response of aged synaptosomes was not impaired by IL-1β_{low} when synaptosomes were preincubated with EM163 or IL-1ra ($P < 0.05$ vs. basal; Fig. 6 *E* and *F*).

Together, our data from synaptosomes show that an age-dependent increase in the AcP/AcPb ratio potentiates the IL-1β–induced suppression of cLTP directly at synapses. Consistent with our hypothesis that increased activation of the AcP-dependent branch of IL-1β signaling drives the age-related heightened sensitivity to impairment by IL-1β, the cLTP deficit in middle-aged animals was rescued by a TIR-domain blocker, which specifically inhibits AcP-dependent signaling. Based on our data and the demonstration that the TIR mimetic AS1 blocks brain IL-1β signaling in vivo (47), we reasoned that AS1 may protect memory function in the aged animal from impairment after acute inflammation.

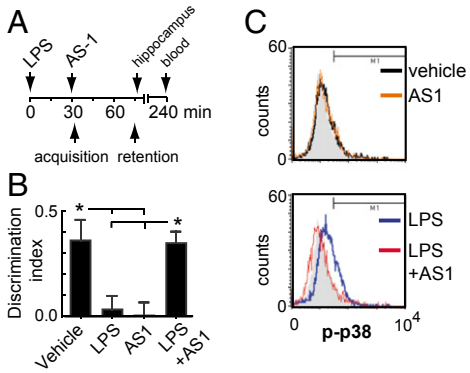

Fig. 7. TIR (IL-1R1–AcP)-dependent IL-1β signaling mediates the memory impairment induced by LPS in old mice. (*A*) Experimental design: 20- to 22-mo-old mice were injected with saline or LPS (0.3 mg/kg, i.p.) and 30 min later with AS1 (200 mg/kg, i.p.) or an equivalent volume of DMSO. OLM task was undertaken immediately after AS1 injection. The acquisition trial (5 min) was followed 45 min later by the retention test trial (5 min). All mice spent equal time exploring both objects in the acquisition trial, indicating no preference for either location ($P = 0.40$; ANOVA). Mice were euthanized immediately after test trial to obtain the hippocampus. (*B*) Discrimination index ($n = 7$ per group). *$P < 0.05$ (ANOVA, Tukey's post hoc test). Data are presented as mean ± SEM. (*C*) Levels of p-p38 (Thr-180/Tyr-182) in size-gated hippocampal synaptosomes from mice used for OLM task. Background fluorescence was determined by using a PE-conjugated control isotype antibody (gray filled histogram) ($n = 4$ per group). $P < 0.01$, LPS vs. LPS+AS1 (Kolmogorov–Smirnov test).

Acute Inflammation Impairs Hippocampal-Dependent Memory in Aged Mice via TIR Domain Signaling. To determine whether the AcP-dependent branch of IL-1β signaling is responsible for the cognitive impairing effects of acute inflammation in aged animals (6, 7, 9, 11), mice were treated with the TIR mimetic AS1 after an acute immune challenge. We challenged 20- to 22-mo-old mice with bacterial LPS (0.3 mg/kg, i.p.) based on previous reports that have consistently shown spatial memory impairments and increased central IL-1β induction by LPS in 18- to 24-mo-old mice (9, 11). Thus, aged mice were injected with LPS or saline, followed 30 min later by administration of the TIR mimetic AS1 immediately before the acquisition trial of the object location memory (OLM) task (50) (Fig. 7*A*). In the retention trial (45 min after acquisition), saline-treated animals exhibited a preference for the novel location over the familiar location (calculated as the discrimination index; $P < 0.05$; Fig. 7*B*), demonstrating intact spatial memory. In contrast, animals challenged with LPS exhibited significantly reduced discrimination indices compared with saline-treated controls ($P < 0.05$; Fig. 7*B*). Consistent with our prediction, administration of the TIR mimetic AS1 prevented the OLM impairment, demonstrating that blocking the AcP-dependent branch of IL-1β signaling prevents LPS-induced spatial memory deficits in aged mice ($P < 0.05$ vs. LPS; Fig. 7*B*). Interestingly, administration of AS1 in the absence of LPS treatment also impaired OLM ($P < 0.05$ vs. vehicle; Fig. 7*B*). This finding is consistent with the notion that endogenous IL-1β at physiologically low levels may be essential for hippocampal memory function (51–53).

Because p38 is a pivotal stress kinase downstream of the AcP-dependent pathway (19) by which IL-1β inhibits hippocampal-dependent memory (54), LTP (29, 55), and BDNF signaling (29), we assessed p38 activation in size-gated synaptosomes from aged mice treated in accordance with the design used to evaluate OLM. Flow cytometry analysis showed that LPS significantly increased p-p38 levels in hippocampal synaptosomes. Importantly, synaptosomes from mice treated with AS1 after LPS challenge showed p-p38 levels comparable to saline-treated mice (Fig. 7*C*), a finding consistent with the rescue of hippocampal-dependent memory by AS1.

Together, our data provide a mechanism for the impairment of hippocampal-dependent memory after immune activation in aged animals, with a key role of AcP-dependent IL-1β signaling and its downstream effector p38 acting directly at synapses.

Discussion

In the hippocampus, aging and inflammation interact to induce memory deficits via IL-1β (6, 7). We demonstrate a previously unidentified mechanism to explain the increased hippocampal sensitivity to IL-1β with age. Our analysis at the synapse level together with in vivo behavior indicate that AcP-dependent signaling is a key pathway mediating the age-related impairment of synaptic plasticity and memory by IL-1β and that synapses themselves are the site of the potentiated IL-1R1–AcP response. We further identify TIR domains as a drug target to selectively block the sensitized IL-1R1–AcP signaling and demonstrate that administration of TIR mimetics can prevent IL-1β–driven impairment of synaptic function and hippocampal-dependent memory in aged animals.

We had hypothesized that chronically elevated IL-1β signaling, such as occurs with age and neurodegenerative disease, can affect the IL-1 receptor system directly in neurons. Here we demonstrate that, along with up-regulating IL-1R1, IL-1β also increases expression of AcP (but not the splice variant AcPb) in hippocampal cultured neurons, thus modulating the relative expression of AcP and AcPb, which are gatekeepers driving different functional outcomes of IL-1β signaling (AcP, proinflammatory, vs. AcPb, neuroprotective). We show that the IL-1β–induced up-regulation of IL-1R1–AcP potentiates the suppression by IL-1β of BDNF signaling in hippocampal neurons, such that BDNF signaling can subsequently be suppressed by exposure to low-level, normally subthreshold IL-1β challenge. The key role of AcP in the potentiated IL-1β signaling was established directly by shRNA knock down of AcP and was supported by AcPb overexpression to reduce AcP interaction with IL-1R1, both of which reduced the IL-1β impairment of BDNF-dependent signaling.

In addition to suppressing BDNF signaling, IL-1β influences a variety of neuronal properties and functions (e.g., excitability, transmitter release) via multiple biochemical pathways (16, 19, 56, 57), which might also be modulated by the relative expression of AcP and AcPb. For instance, it has been shown that high IL-1β concentrations (1–10 nM) activate p38 and require AcP, whereas low IL-1β concentrations (≤0.6 pM) may improve neuronal activity via AcPb-dependent activation of Src kinase (22). Our finding that AcP and AcPb compete for IL-1R1 binding suggests that the relative expression of these IL-1 coreceptors may change the effective concentration at which IL-1β switches from Src to p38 activation. Indeed, we found that a low IL-1β concentration (3 pM) induced the activation of p38 in neurons with an elevated AcP/AcPb ratio. Thus, our data suggest that chronically elevated IL-1β signaling can shift the effects of low IL-1β concentrations from facilitating neuronal activity via Src (22) to suppression of BDNF signaling (29) and synaptic plasticity (29, 55) via p38.

Previous reports have shown that hippocampal-dependent memory is vulnerable to IL-1 after infection-like immune challenges in aged animals (6, 7), and it has recently been found that IL-1 signaling also mediates the age-related cognitive decline associated with low-grade sterile inflammation, an innate immune response induced by the accumulation of endogenous "danger signals" in the absence of overt infection (3). Because intact synaptic activity is essential for learning and memory, we explored the hypothesis that IL-1β directly impacts synapse functionality. Using synaptosomes isolated from the hippocampus of young and middle-aged animals, we demonstrated that the AcP/AcPb ratio in synapses shifts with age, with a nearly threefold

increase in the ratio in middle-aged synapses. Paralleling the neuronal culture data, we found that an increased AcP/AcPb ratio is associated with a sensitized IL-1β response with increasing age. FASS-LTP revealed that low levels of IL-1β (3 pM) did not impair cLTP-induced changes in synaptosomes isolated from young mice, but totally suppressed the cLTP response in synaptosomes from middle-aged mice. Thus, aging biased IL-1β signaling toward AcP-dependent (proinflammatory) responses directly at hippocampal synapses and thereby impaired LTP. Our data are consistent with the early impairments of hippocampal-dependent memory associated with deficits in CA1 late LTP (45) and with increased hippocampal expression of inflammatory genes (58) in middle-aged mice. Moreover, middle-aged mice also exhibit an increased Nlrp3 inflammasome-dependent activation of caspase-1 (IL-1–converting enzyme), a significant finding because Nlrp3 inflammasome is a major immune sensor, causing age-related sterile inflammation via caspase-1 and IL-1β in both periphery and CNS (3). We suggest that chronically elevated brain IL-1β levels generate increased sensitivity to IL-1β at the synapse, which may be an early factor driving hippocampal dysfunction.

Our data, together with the literature, indicate that inflammation wields a double-edged sword in the aged brain, not only exaggerating microglia responses (15) to immune challenge, but also heightening IL-1β sensitivity at the synapse. Consistent with the in vitro finding that microglia-derived IL-1β can reduce spine density (59), our data demonstrate that hippocampal synapses contain the biochemical IL-1β–signaling pathway(s) impairing plasticity (e.g., p38) (29, 54). To our knowledge, we demonstrate for the first time that AcP-dependent IL-1β signaling at the synapse can itself underlie the suppression of plasticity by IL-1β. The synaptic response to IL-1β is amplified with age, and IL-1β can suppress LTP in the aged brain by acting directly on sensitized synapses, an effect that is not dependent on an intermediary response from IL-1β–activated glia. Our results are consistent with the interaction of IL-1β and aging on LTP previously identified in more complex systems (e.g., hippocampal slices and in vivo) (4, 12) and extend the growing understanding that synaptic dysfunction is a major cause of cognitive decline in aging (60) and many brain diseases (61), including Alzheimer's disease (AD) (62).

One of our goals was to identify therapeutic interventions that can counteract the cognitive impairments caused by IL-1β in the aged animal. Our data identify AS1, a TIR mimetic that blocks MyD88 recruitment to TIR domains on the IL-1R1–AcP receptor and prevents IL-1R1–AcP signaling (47), as such a therapeutic agent. AS1 inhibited the following: (*i*) the potentiated IL-1β inflammatory response that suppresses BDNF signaling in cultured hippocampal neurons; (*ii*) the age-related impairment of cLTP by IL-1β in synaptosomes; and (*iii*) the impairment of hippocampal-dependent memory and p38 activation that occur in aged animals after peripheral immune challenge with LPS. In vitro experiments demonstrate a direct action of AS1 on neurons and synapses; however, the beneficial effects of AS1 on memory and p38 activation in animals challenged with LPS might be reflecting the inhibition of IL-1β signaling in many cell types in the periphery and in the brain, including neurons and glia.

Overall, we provide, to our knowledge, the first evidence that the IL-1–receptor system undergoes age-related reconfiguration in neurons. We demonstrate that the IL-1–receptor subunit reconfiguration sensitizes the IL-1R1–AcP-dependent response, occurs directly at synapses, and renders synaptic plasticity and memory vulnerable to impairment by IL-1β challenge. It stands to reason that the increased IL-1R1–AcP receptor sensitivity at the synapse is likely a common mechanism increasing neuronal vulnerability in brain diseases associated with IL-1β–driven neuroinflammation, such as depression (63), chronic stress (64), and AD (65). Finally, supporting the notion that the relative abundance of AcP and its splice variant AcPb modulates the strength and direction of neuronal IL-1β signaling, our work opens a road toward the search for selective therapeutic strategies to alleviate cognitive decline in the elderly population.

Methods

A complete description of experimental procedures is available in *SI Methods*.

Animals. Both mice and rats were housed with food and water ad libitum. Lights were maintained on a 12:12 light/dark cycle, and behavior testing was carried out during the light phase of the cycle. All procedures used in the present study followed the Principles of Laboratory Animal Care from the NIH and were approved by the University of California, Irvine, Institutional Animal Care and Use Committee.

Hippocampal Cell Cultures. Primary cultures were prepared from embryonic day 18 (E18) Sprague–Dawley rats as described (27). After 5–7 DIV, neurons were treated at 37 °C, O_2/CO_2 (95%/5%). Immunostaining in cell cultures was performed as described (27). The fluorescence analysis was performed in RFP-positive cells. It should be noted that we used E18 rats for all culture studies and mice for all other experiments to open the possibility of follow-up studies in transgenic models. Rat cultures provided great quantities of tissue for concentration–response, transgene-expression, and signaling-pathway studies and other large pilot culture experiments. We, however, did confirm IL-1β sensitivity after IL-1β pretreatment in cultured neurons from E16 mice. It is also noteworthy that the localization of IL-1R1 and AcP in the mouse brain is similar to that observed in the rat brain (66) and that IL-1β similarly activates p38 in primary hippocampal neurons from rat (67, 68) and mouse (22).

Biochemistry. Homogenates (from neuronal cultures, tissue, and hippocampal synaptosomes) and immunoblotting were prepared as described (27). Immunoprecipitation was performed with a commercially available kit (Active Motif). Antibodies are listed in Table S1. Quantitative densitometric analyses were performed on images of immunoblots with ImageJ (National Institutes of Health). Total RNA extraction, cDNA synthesis, and qPCR were performed with commercially available kits (Qiagen).

Transfections. *AcP* knockdown and *AcPb* overexpression were performed by using Cellecta lentiviral particles encoding RFP as reporter. Neurons were infected at 3 DIV according to the manufacturer's protocol. For *AcP* knockdown, we used vectors containing either a negative control shRNA sequence or a shRNA to target rat *AcP* mRNA. Control–shRNA (targeting luciferase, shLuc): target sequence, 5′-CGCTGAGTACTTCGAAATGTC-3′; insert (hairpin) sequence, 5′- ACCGGCGCTGAGTACTTTGAAATGTTGTTAATATTCATAGCGACATTTCGAAGTACTCAGCGTTTT -3′; construct, pRSI16-U6-**shLuc**-UbiC-TagRFP-2A-Puro. AcP-shRNA: target sequence, 5′-GAGACCCTGAGCTTCATTCAG-3′; insert sequence 5′-ACCGGGAGACCCTGAGTTTCATTTAGGTTAATATTCATAGCCTGAATGAAGCTCAGGGTCTCTTTT-3′; construct, pRSI16-U6-**shAcP**-UbiC-TagRFP-2A-Puro (*SI Text*). For AcPb overexpression experiments, vectors contained either the rat AcPb sequence (RefSeq: GU123169.1; vector: pR-CMV-**AcPb**-EF1-TagRFP) or an empty vector as control. Sequencing quality-control data verified the identity of AcPb rat gene.

Synaptosome Preparation by Ficoll/Sucrose Gradient. Synaptosomes from B6129SF2/J mice were prepared by using a discontinuous Ficoll gradient as described (69).

RNA Extraction and qPCR. Total RNA extraction from rat neuronal cultures, mouse (B6129SF2/J) whole brain (no cerebellum), and mouse (B6129SF2/J) hippocampal synaptosomes was performed by using TRIzol (Molecular Research Center) and the Qiagen RNeasy Mini Kit. The RNA Integrity Number of RNA samples was ≥7, as evaluated by the Genomics High-Throughput Facility at the University of California, Irvine. In neuronal cultures, gene expression was quantified by using TaqMan assays (Applied Biosystems) using appropriate primer sets and probes, as listed in Table S2 [AcP (21), AcPb (21), IL-1R1 (70), and GAPDH (70)]. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method and expressed as fold change over control after normalizing with control samples. In mouse brain, qPCR was performed by using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). Primer sets (71) are described in Table S3. Data were analyzed by the $2^{-\Delta\Delta Ct}$ method and expressed as fold change over young mice (6 mo) after normalizing with input samples.

FASS-LTP. Fresh hippocampal synaptosome P2 fractions were obtained from B6129SF2/J mice (42) and stimulated (cLTP) as described (72–74). Briefly, hippocampi were rapidly dissected from a single mouse and homogenized in 320 mM sucrose containing Hepes (10 mM) and protease/phosphatase inhibitors mixture (Pierce) at pH 7.4. The homogenate was centrifuged at 1,200 × *g* (10 min). Supernatant was transferred into two microfuge tubes and centrifuged at 13,000 × *g* (20 min). Supernatants (S2) were removed, and pellets (P2 crude synaptosome fraction) were resuspended by gently pipetting up and down in 1.5 mL of extracellular (tube 1) or cLTP (tube 2) solutions. Extracellular solution contains (in mM): 120 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 15 glucose, and 15 Hepes, pH 7.4; whereas cLTP solution is Mg^{2+}-free and contains (in mM): 150 NaCl, 2 $CaCl_2$, 5 KCl, 10 Hepes, and 30 glucose, pH 7.4 (73). Synaptosome P2 fractions were incubated in a cell culture dish (30 mm) with agitation at room temperature for 15–30 min for recovery. After recovery, synaptosomes maintained in cLTP solution were supplemented with 0.001 mM strychnine and 0.02 mM bicuculline methiodide, and 180 μL (50–100 μg of protein determined by BCA assay) of this suspension was transferred to cytometry tubes. As control, an equal volume (180 μL) of synaptosome maintained in external solution was also transferred to a cytometry tube. Synaptosomes in external solution were used to determine basal levels of potentiated synaptosomes (see below). For stimulation, 20 μL of external solution was added to control synaptosomes (in external solution), whereas 20 μL of glycine (5 mM in cLTP solution) was added to synaptosomes in cLTP solution (final [glycine] = 500 μM; ref. 74). After 15 min of glycine treatment, synaptosomes were depolarized with 200 μL of a [high] KCl solution consisting of (in mM): 50 NaCl, 2 $CaCl_2$, 100 KCl, 10 Hepes, 30 glucose, 0.5 glycine, 0.001 strychnine, and 0.02 bicuculline methiodide, pH 7.4 (final [KCl] = 50 mM) and incubated for 30 min; 200 μL of external solution were added to control synaptosomes. For cytokine treatment, 3 pM IL-1β was added 5 min before glycine, whereas EM-163 or IL-1ra was added 10 min before IL-1β in the corresponding tubes. After KCl incubation, stimulation was stopped by sequential addition of 0.5 mL of ice-cold 0.1 mM EGTA–PBS (pH 7.4) and 4 mL of ice-cold blocking buffer [5% (wt/vol) FBS in PBS]. Tubes were chilled on ice and centrifuged at 2,500 × *g* for 5 min at 4 °C (Sorvall RT6000B). After centrifugation, the pellet was resuspended and incubated with primary-antibody solution contained rabbit anti-GluR1 and mouse anti-Nrx1β antibodies (listed in Table S1), both at a concentration of 2.5 μg/mL in blocking buffer. After incubation, synaptosomes were washed (×2) with 4 mL of ice-cold blocking buffer, centrifuged (2,500 × *g* for 5 min at 4 °C), and pellet-resuspended in secondary-antibody solution [400 μL; anti-rabbit–Alexa 488 and anti-mouse–Alexa 647 antibodies (Life Sciences), both at 2.5 μL/mL]. After 30 min on ice with agitation and protected from light, synaptosomes were washed as described and resuspended in 400 μL of 2% paraformaldehyde in PBS. Samples were acquired by using a Becton Dickinson FACSCalibur flow cytometer (BD Biosciences). Relative size and granularity was determined by forward scatter (FSC) and side scatter (SSC) properties. FSC, SSC, and fluorescence [FL1 (530 ± 15 nm) and FL4 (650 ± 25 nm)] signals were collected by using log amplification. Identical FSC settings were used for acquiring data on bead standards and samples. Small fragments and debris were excluded by establishing a FSC-H threshold (325). Synaptosome integrity was assessed with Calcein AM by using a FL1 detector. A total of 10,000 size-gated particles were collected and analyzed for each sample by using CellQuest Pro software (BD Biosciences).

OLM Test, p-p38 Detection in Synaptosomes, and Cytokine Quantification. Mouse (C57BL/6J) cognition was evaluated by using the OLM test, adapted from described tasks (50). The relative exploration time was recorded and expressed as a discrimination index [$DI = (t_{\text{novel}} - t_{\text{familiar}})/(t_{\text{novel}} + t_{\text{familiar}})$]. All animals were euthanized immediately after OLM testing, and hippocampi were removed and immediately processed for Western blot or flow cytometry assays. Immunolabeling for p-p38 detection by flow cytometry in synaptosomes was performed according to a method for staining of intracellular antigens. Cytokines in plasma were quantified by Multiplexing LASER Bead Technology (Eve Technologies).

Statistical Analysis. Sample sizes were chosen on the basis of pilot experiments (FASS-LTP) and previous experience with similar types of in vitro (27, 28) and in vivo (75) experiments. Mann–Whitney test (two-tailed) was used as nonparametric *t* test for unpaired data. ANOVA (parametric test) was used where assumptions of normality (Kolmogorov–Smirnov) and equal variance (Bartlett's test) were met and was replaced by Kruskal–Wallis (nonparametric test) where appropriate. One-way ANOVA was followed by post hoc Tukey's test for mean comparisons of three or more groups, whereas Kruskal–Wallis was followed by Dunn's post hoc test. Two-way ANOVAs were followed by Bonferroni's post hoc test. Statistical tests and the nonlinear fit shown in Fig. 1*C* were performed by using GraphPad Prism (Version 5.0). In Fig. 7*D*, sample distribution comparison was performed by the Kolmogorov–Smirnov test using CellQuest Pro software. Data are presented as mean ± SEM. $P < 0.05$ was considered significant.

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