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Longitudinal noninvasive monitoring of transcription factor activation in cardiovascular regulatory nuclei using bioluminescence imaging

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Peterson JR, Infanger DW, Braga VA, Zhang Y, Sharma RV, Engelhardt JF, Davisson RL. Longitudinal noninvasive monitoring of transcription factor activation in cardiovascular regulatory nuclei using bioluminescence imaging. *Physiol Genomics* 33: 292–299, 2008. First published January 29, 2008; doi:10.1152/physiolgenomics.00296.2007.—The ability to monitor transcription factor (TF) activation in the central nervous system (CNS) has the potential to provide novel information regarding the molecular mechanisms underlying a wide range of neurobiological processes. However, traditional biochemical assays limit the mapping of TF activity to select time points. In vivo bioluminescence imaging (BLI) has emerged as an attractive technology for visualizing internal molecular events in the same animal over time. Here, we evaluated the utility of BLI, in combination with virally mediated delivery of reporter constructs to cardiovascular nuclei, for monitoring of TF activity in these discrete brain regions. Following viral gene transfer of NF-κB-driven luciferase reporter to the subfornical organ (SFO), BLI enabled daily measurements of baseline TF activity in the same animal for 1 mo. Importantly, systemic endotoxin, a stimulator of NF-κB activity, induced dramatic and dose-dependent increases in NF-κB-dependent bioluminescence in the SFO up to 30 days after gene transfer. Cotreatment with a dominant-negative IκBα mutant significantly prevented endotoxin-dependent NF-κB activation, confirming the specificity of the bioluminescence signal. NF-κB-dependent luminescence signals were also stable and inducible 1 mo following delivery of luciferase reporter construct to the paraventricular nucleus or rostral ventrolateral medulla. Lastly, using targeted adenoviral delivery of an AP-1 responsive luciferase reporter, we showed similar baseline and endotoxin-induced AP-1 activity in these same brain regions as with NF-κB reporters. These results demonstrate that BLI, in combination with virally mediated gene transfer, is a powerful method for longitudinal monitoring and quantification of TF activity in targeted CNS nuclei in vivo.

luciferase; nuclear factor-κB; activator protein-1; subfornical organ; paraventricular nucleus; rostral ventrolateral medulla; viral gene transfer

THE MAJORITY OF LONG-LASTING neurobiological processes require structural and functional changes in neuronal networks that are ultimately dependent upon changes in gene expression. For example, the progression of chronic cardiovascular diseases such as hypertension and heart failure is driven by sustained dysregulation of cardiovascular regulatory networks in the central nervous system (CNS) (38). Since the mechanisms underlying long-term changes in neuronal activity within these networks may involve shifts in transcription factor activity (30), a complete spatio-temporal portrait of relevant transcription factor activation patterns has the potential to fundamentally advance our knowledge of the pathophysiology of cardiovascular diseases and other chronic conditions of CNS dysfunction.

Longitudinal mapping of transcription factor activation patterns in CNS nuclei has been challenging, primarily due to the constraints of traditional biochemical assays. Because classic methods require the death of numerous cohorts of animals to achieve sufficient tissue and multiple time points, they provide only a limited glimpse of the time course of transcription factor activity and also suffer from potential variability between different groups of animals. Furthermore, conventional assays focus on the increased binding potential or nuclear translocation of transcription factor subunits, failing to provide direct information regarding changes in gene transcription.

In vivo bioluminescence imaging (BLI) has emerged as a powerful tool for the real time and noninvasive study of a wide range of biological processes (12). Though firefly luciferase is a routinely utilized reporter gene in in vitro assays, recent advances in live animal imaging have enabled the visualization of firefly luciferase-dependent luminescence in discrete tissues of living animals. This strategy has provided valuable new information about spatio-temporal patterns of a number of physiologic and pathophysiological events, including tumor growth and metastasis, progression of microbial infection, and stem cell tracking (9, 10). Recently, the use of in vivo BLI has been extended to the study of the CNS. In the brain, BLI has been employed in applications that parallel its use in the periphery, providing a novel approach for tracking tumor growth, monitoring of herpes simplex virus infection and therapy, and visualizing migration patterns of neural progenitor cells (8).

Though the majority of studies of transcription factor activation utilizing in vivo BLI have focused on events in the periphery, a recent study by Luo et al. (22) utilized transgenic mice globally expressing Smad2-dependent luciferase to demonstrate central Smad2 activation in response to injury with the excitotoxin kainic acid. Although this study indeed demonstrated transcription factor activation in the brain using BLI, spatio-temporal localization of the bioluminescence signal in these animals with global expression of a luciferase reporter was not possible. Thus, with global transgenic animals, ex vivo studies remain necessary to localize the precise source of the bioluminescence signal in the CNS.

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To utilize BLI for the monitoring of transcription factor activation in specific brain regions in vivo, expression of the luciferase reporter construct must be restricted to CNS areas of interest. The use of viral vectors is a well-established tool for targeted expression of transgenes in distinct CNS nuclei (14, 35, 36). Indeed, we and others have used this strategy to dissect out the molecular mechanisms utilized by specific neuro-cardiovascular networks of the brain. Here, we sought to determine the feasibility of utilizing in vivo BLI, coupled with viral delivery of luciferase reporters to the CNS, for the longitudinal mapping of transcription factor activity in specific cardiovascular regulatory nuclei in vivo. To achieve this, we examined basal and systemic endotoxin-induced activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) in three discrete cardiovascular regulatory nuclei, including the subfornical organ (SFO), the paraventricular nucleus (PVN) of the hypothalamus, and the rostral ventrolateral medulla (RVLM). Each of these regions plays an important role in blood pressure, cardiorenal, and fluid homeostasis. These studies establish the power of BLI, coupled with targeted adenovirally mediated gene transfer to CNS cardiovascular nuclei, as an innovative tool for the longitudinal quantification of transcription factor activity in these distinct CNS nuclei in vivo.

METHODS

Viral vectors. Recombinant E1-deleted adenoviral vectors encoding the firefly luciferase gene driven off the human cytomegalovirus promoter (AdCMVluc) or response elements specific for activated NF-κB (AdNF-κBluc) (34) were obtained from the University of Iowa Gene Transfer Vector Core (Dr. Beverley Davidson). Construction of AdNF-κBluc has been described previously (34). Briefly, a fragment of pNF-κB-Luc plasmid (Clontech Laboratories, Palo Alto, CA) containing the luciferase gene driven by four tandem copies of the NF-κB consensus sequence fused to a TATA-like promoter from the herpes simplex virus-thymidine kinase gene was inserted into a promoterless adenoviral shuttle plasmid (pAd5mcspA), and AdNF-κBLuc virus was generated by homologous recombination. Adenovirus containing an AP-1 responsive luciferase expression cassette (AdAP-1luc) was generated by previously described methods (1, 39). Briefly, a fragment from the pAP1(PMA)-TA-Luc plasmid (Clontech Laboratories, Palo Alto, CA) containing the firefly luciferase gene, driven by six tandem copies of the AP-1 enhancer linked to the minimal TATA box promoter from the herpes simplex virus-thymidine kinase gene was inserted into a promoterless adenoviral shuttle plasmid (pAd5mcspA), and AdAP-1Luc virus was generated by homologous recombination. Purified high-titer stocks of AdAP-1luc were generated by two sequential rounds of $CsCl_2$ banding and desalted by gel filtration in phosphate-buffered saline on a Sephadex G-50 column prior to use. Construction of adenovirus encoding a dominant negative isoform of the NF-κB regulatory subunit IκBα (AdS32/S36A IκBα) has been described previously (18, 34).

Targeted gene transfer of luciferase constructs to CNS nuclei. For studies targeting the subfornical organ, adult C57BL/6 mice (Harlan, Indianapolis, IN) were injected intracerebroventricularly (ICV, 500 nl) as previously described (35, 36) with AdCMVluc (3×10^{10} pfu/ml), AdNF-κBluc (1×10^{11} pfu/ml), or AdAP-1luc (1×10^{11} pfu/ml). Separate groups of mice were coinfected (ICV) with AdNF-κBluc and either AdS32/S36A IκBα (2×10^{11} pfu/ml) or AdLacZ (4×10^{10} pfu/ml). It should be noted that the total concentration and volume of adenovirus given in coinfection experiments were equal to that in the single adenoviral infection studies. For studies targeting the PVN or RVLM, mice were microinjected as described (35, 36) with AdNF-κBluc or AdAP-1luc to the PVN or RVLM, using the following coordinates (relative to bregma, 200 nl bilaterally): PVN, 0.7 mm caudal, 0.3 mm either side of midline, 4.5 mm ventral; RVLM, 6.5 mm caudal, 1.2 mm either side of midline, 5.7 mm ventral. All procedures met or exceeded the guidelines set forth by the National Institutes of Health and were approved by the University of Iowa and Cornell University Animal Care and Use Committees.

Endotoxin administration. Lipopolysaccharides isolated from *Escherichia coli* 0111:B4 (Sigma L2630) were suspended in saline at 5 mg/ml. For dose-response experiments, mice were injected with 200 μl (ip) of appropriately diluted solution to produce 1, 4, 8, or 40 μg/g dosages. Following injections, mice were left undisturbed for 4 h prior to imaging.

In vivo bioluminescence imaging. All in vivo bioluminescence images were obtained with the IVIS 200 instrument (Xenogen). Following D-luciferin (150 mg/kg ip, Xenogen) injection, mice were transferred to a light-sealed imaging cabinet and placed under isoflurane anesthesia (2% in oxygen). Using a charge-coupled device (CCD) camera with exposure times of 1–2 min, images were obtained at 1–5 min intervals for kinetics experiments and at 10–15 min following luciferin injection for all other experiments. Data were analyzed and signal intensity quantified with Xenogen Living Image Software (Living Image, v2.60.1).

Ex vivo bioluminescence imaging. To further confirm that bioluminescence detected in vivo was restricted to the brain region targeted, ex vivo BLI was performed in a subset of mice 4 h after treatment with LPS at 30 days postgene transfer of AdAP-1luc to either the PVN or RVLM. Following D-luciferin (150 mg/kg ip, Xenogen) injection, in vivo bioluminescence images were obtained as described. Mice were then decapitated, and fresh brains were immediately removed from the skull and sectioned (coronal, 1 mm). Sections from PVN and RVLM regions were then placed inside the IVIS 200 instrument and imaged using a CCD camera with exposure times of 1–2 min.

Data and statistical analyses. Results are expressed as means ± SE. Data were analyzed by Student's *t*-test for comparisons between groups or ANOVA followed by the Tukey test for multiple comparisons. Statistical analyses were performed using Prism (GraphPad Software, Inc).

RESULTS

Establishing the optimal parameters for measuring bioluminescence in cardiovascular regulatory nuclei in the mouse brain. In vivo BLI has recently been employed as a novel strategy for the investigation of a wide variety of biological processes (8). However, information about the kinetics of the reaction between luciferase and its substrate luciferin in the context of specific brain regions is lacking. We first sought to verify the kinetics of the bioluminescence signal following delivery of AdCMVluc to the SFO as the prototype. Adult C57BL/6 mice underwent SFO-targeted gene transfer of AdCMVluc, the day after which serial imaging commenced using the Xenogen IVIS 200. Predictably, following injection of luciferin (150 mg/kg ip) the bioluminescence signal rose within minutes, peaked at 10–15 min, and then decayed 90% by 120 min, returning to baseline by 240 min (Fig. 1*A*). These results confirm previous reports indicating that reliable measurements of the peak luminescence signal in the CNS are obtained at 10–15 min following luciferin administration and that the signal returns to baseline within 4 h (6, 11, 16). Although 150 mg/kg luciferin is the standard dose used (11, 16), we did try higher doses of luciferin (300, 600 mg/kg ip). These high doses increased photon flux; however, the amount of variability was high (data not shown). Furthermore, the potential for toxicity at these higher doses led us to carry out all

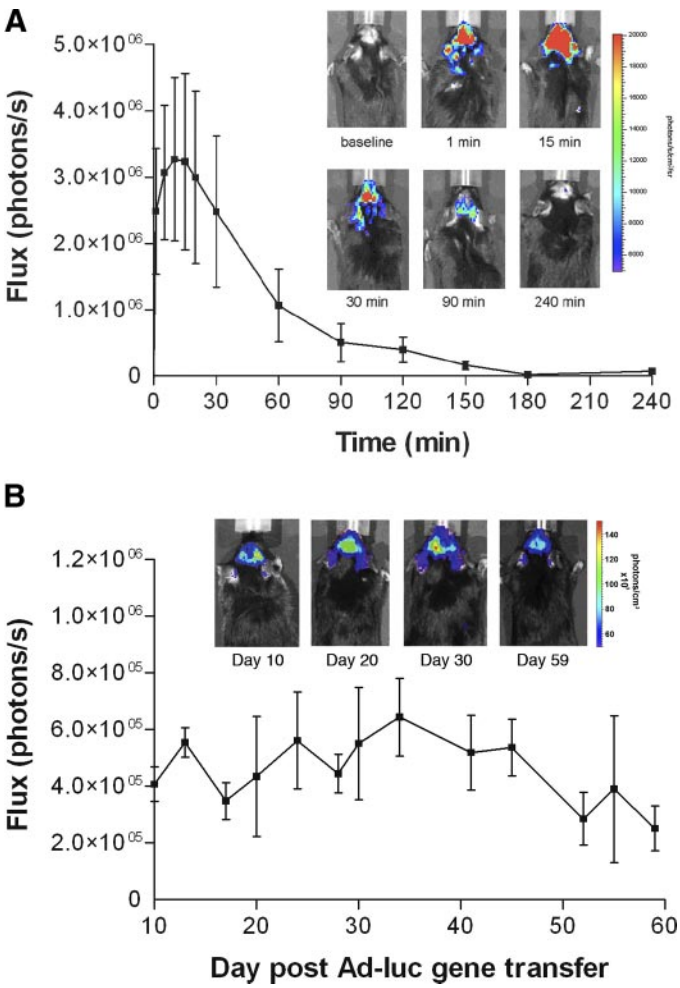

Fig. 1. Kinetics and long-term stability of the bioluminescence signal following adenovirus-mediated gene transfer of cytomegalovirus (CMV)-driven luciferase to the subfornical organ (SFO). *A*: time-dependent decay of bioluminescence signal following luciferin administration (150 mg/kg ip) in mice 7 days following SFO-targeted gene transfer of AdCMVluc (n = 4). Detectable luminescence (flux; photons/s) peaked 10–15 min following luciferin injection and decayed 90% by 4 h. *Inset images*: representative bioluminescence overlays prior to (baseline) and at defined time points following luciferin injection. Areas of highest photon detection are shown in red, and lower levels of detection are shown in blue. *B*: AdCMVluc-mediated bioluminescence remains stable and detectable for up to 2 mo after targeted gene transfer to the SFO. Following SFO-targeted gene transfer of CMV-driven luciferase (n = 5), luminescence was measured 10 min after luciferin administration (150 mg/kg ip) every 3–7 days over the course of 2 mo. *Inset*: representative pseudo-color bioluminescence overlays at defined time points postgene transfer.

subsequent studies at the 150 mg/kg dose. It should be noted that in naïve mice, i.e., animals that have not undergone gene transfer of luciferase reporters, bioluminescence measured at the skull is equivalent before and after administration of luciferin (data not shown). This background bioluminescence is negligible (i.e., <500 photons/s) compared with the signal obtained in mice following virally mediated gene transfer of luciferase reporters to the CNS (e.g., 3×10^6 photons/s).

Next, to establish the long-term stability of the bioluminescence signal following SFO-targeted delivery of luciferase, BLI was performed intermittently in this same group of animals over the span of 2 mo. There was a brief, transient peak in signal 2 days after gene transfer ($2.3 \pm 0.1 \times 10^6$ photons/s, n = 5), but this signal was stable between *day 10* and *day 60* (Fig. 1*B*). In addition to being stable, the bioluminescence signal also remained quite robust. It should be noted that the transient peak in luciferase activity immediately following gene transfer is likely due to immune mechanisms as well as promoter silencing typical of adenoviral gene transfer to the CNS (5, 29). Importantly, these data indicate that adenovirally mediated expression of luciferase in the mouse CNS remains stable over a period of time sufficient for extended physiological studies.

Longitudinal monitoring of NF-κB activation in cardiovascular regulatory nuclei following adenovirally mediated gene transfer. Having established the feasibility and optimum parameters for monitoring the bioluminescence signal following adenoviral gene transfer to the SFO, we next sought to verify this method for spatio-temporal mapping of transcription factor activation in this brain region. We first focused on NF-κB, a transcription factor that plays an important role in a number of long-term neurobiological processes (23, 27) and is known to be activated during systemic lipopolysaccharide (LPS) challenge (31, 32). To measure NF-κB activation, we utilized an adenoviral vector containing the luciferase gene downstream of NF-κB consensus sequences (AdNF-κBluc). Following gene transfer of AdNF-κBluc to the SFO, baseline NF-κB activity was measured for 1 mo starting at 10 days following gene transfer. This time point was chosen to minimize potential confounding effects of any postsurgical inflammation-mediated activation of NF-κB. Similar to AdCMVluc results described above, BLI demonstrated highly stable baseline NF-κB activity over the course of the study (Fig. 2*A*). However, it should be noted that levels were much lower (although detectable) than AdCMVluc, an important attribute for being able to detect changes in NF-κB activity. At 30 days following gene transfer, we tested the transactivation potential of the NF-κB-luc construct in the SFO by measuring LPS-induced increases in the bioluminescence signal in the brain. As shown in Fig. 2*A*, LPS treatment (8 μg/g ip) induced a robust and rapid increase in NF-κB-driven luciferase expression in the SFO, as indicated by a nearly 100-fold increase in bioluminescence signal 4 h after LPS injection. Furthermore, LPS induced a dose-dependent increase in NF-κB activation (Fig. 2*B*, 1–40 μg/g ip). Although 40 μg/g LPS produced the highest bioluminescence signal, because of the high mortality associated with this dose, 8 μg/g LPS was used in all subsequent experiments.

To confirm that LPS-induced increases in bioluminescence were due to NF-κB-dependent increases in luciferase expression, a separate group of mice underwent co-infection with an NF-κB inhibitor expressing virus, the S32/36A IκBα suppressor mutant (AdS32/36A IκBα) (18), along with AdNF-κBluc. As shown in Fig. 2*C*, cotreatment with AdS32/36A IκBα caused a >90% inhibition in NF-κB-dependent luminescence following LPS injection, confirming that LPS-induced increases in observed bioluminescence are mediated via NF-κB transactivation in the SFO.

After establishing the ability of BLI to monitor NF-κB activation in the SFO, we next sought to establish this technique in brain regions downstream of the SFO involved in neuro-cardiovascular regulation. Blood-borne molecules such as LPS influence cardiovascular activity by triggering signaling pathways in neurons of CVOs, such as the SFO, and these then project to other key cardiovascular regulatory nuclei both in the forebrain and brainstem (24, 25). A principal target of the SFO is the hypothalamic PVN. Importantly, the PVN plays a pivotal role in mediating the cardiovascular response to systemic LPS, primarily by regulating release of ACTH, oxytocin, and vasopressin (3, 15). Using a similar gene transfer strategy, but this time targeting the PVN, we used BLI to monitor baseline and LPS-induced NF-κB activity. Similar to the SFO, baseline NF-κB-dependent luminescence was low but detectable following adenoviral-mediated gene transfer of NF-κB-luc to the PVN (Fig. 3*A*). Furthermore, LPS-induced a robust increase in NF-κB activation 30 days after gene transfer (Fig. 3*A*).

In addition to the PVN, the RVLM also plays a key role in regulating cardiovascular activity by integrating signals originating in CVOs, the PVN, and other sites, and initiating appropriate alterations in sympathetic outflow (17, 28). To further establish the utility of BLI for monitoring transcription factor activity in this cardiovascular regulatory region, as well as to examine potential relationships between LPS and transcription factor activation in the RVLM, we next used BLI to test the hypothesis that systemic LPS also induces NF-κB transactivation within the RVLM. Similar to our findings in the SFO and PVN, baseline NF-κB activity was detectable and stable out to 30 days following adenovirus-mediated gene transfer of NF-κB-luc to the RVLM (Fig. 3*B*). Also, similar to the SFO and PVN, LPS induced a robust increase in NF-κB-dependent luminescence 4 h after treatment (8 μg/g LPS ip, Fig. 3*B*). Taken together, these results demonstrate the ability of BLI to track NF-κB activity in vivo both at baseline and following systemic LPS challenge along the length of the SFO-PVN-RVLM axis.

Longitudinal in vivo monitoring of AP-1 activation in cardiovascular regulatory nuclei. To determine the applicability of this technology to other transcription factors, we next applied this strategy to visualize CNS site-specific activation of the transcription factor AP-1. Activation of AP-1 within various cardiovascular regulatory nuclei has been implicated in cardiovascular disease (2, 21), and AP-1 is also known to be activated in CNS cardiovascular regulatory sites during systemic LPS challenge (3, 15, 37). Traditional methods of monitoring AP-1 activity in the CNS rely upon c-Fos immunohistochemistry (7) or gel-shift assays to evaluate AP-1 DNA binding (21) and, therefore, are limited to only select time points. To test the ability of BLI for daily in vivo monitoring of AP-1 activity in the CNS, mice underwent SFO-, PVN-, or RVLM-targeted gene transfer with an adenovirus encoding the luciferase gene downstream of AP-1 consensus sequences (AdAP-1luc) and were monitored using BLI for 30 days as described above for NF-κB. As shown in Fig. 4, AP-1-dependent luminescence exhibited low yet detectable basal levels of activity in the SFO, PVN, and RVLM that persisted throughout the experiment. Furthermore, systemic LPS challenge caused a robust increase in AP-1-mediated luminescence in all three CNS nuclei 4 wk after gene transfer.

To confirm correct localization of the AP-1-luciferase reporter, ex vivo luminescence images were obtained of coronal

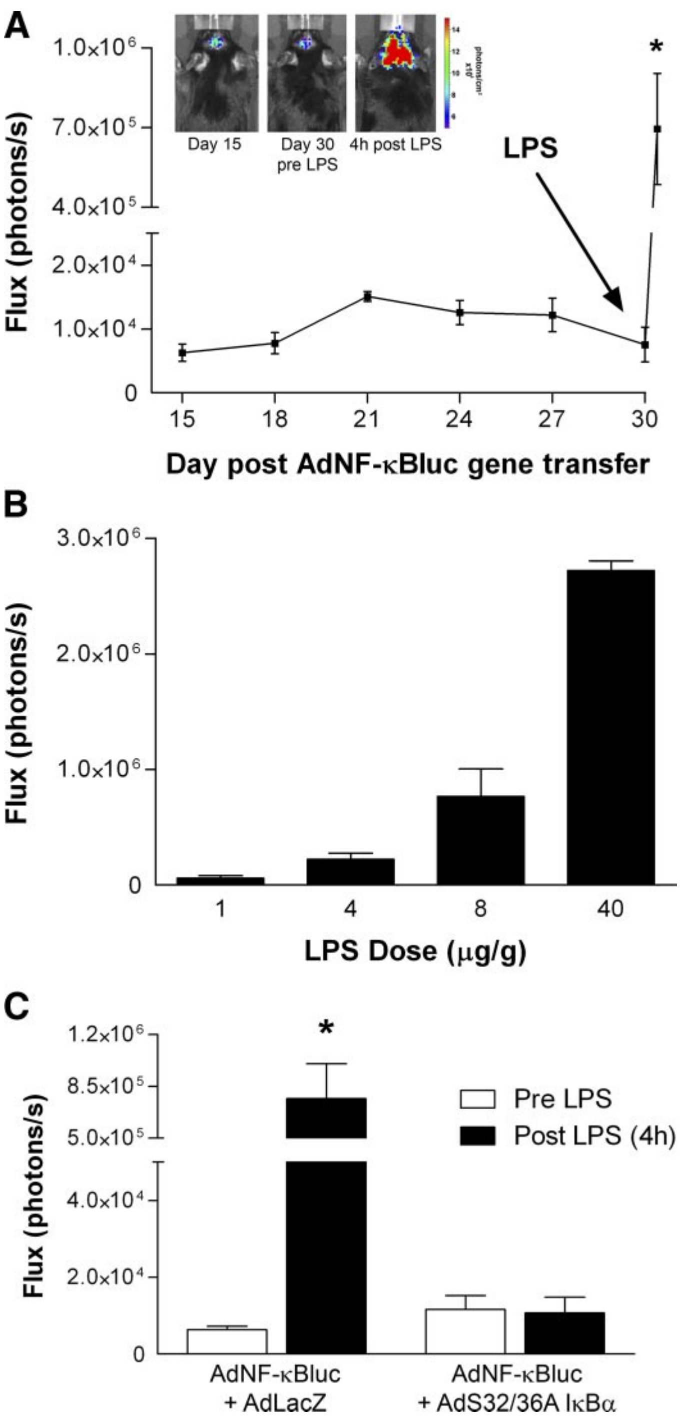

Fig. 2. In vivo bioluminescence imaging (BLI) following viral gene transfer of NF-κB-driven luciferase reporter enables longitudinal monitoring of NF-κB activation. *A*: bioluminescence profile following targeted NF-κB-luc delivery to the SFO ($n = 4$). Average luminescence (flux, photons/s) was monitored every 2–4 days for 1 mo. At 30 days after gene transfer, systemic LPS challenge (8 μg/g ip) induced a dramatic increase in NF-κB-mediated bioluminescence. *Inset*: typical bioluminescence overlays at defined time points. Highest photon emission is displayed in red, and areas of lowest detectable photon emission are displayed in blue. *$P < 0.05$ vs. pre LPS. *B*: LPS induced a dose-dependent increase in NF-κB-mediated bioluminescence in the SFO. At 30 days post-SFO-targeted gene transfer of AdNF-κBluc, peak bioluminescence signals were obtained 4 h after systemic injections of different doses of LPS (1, 4, 8, or 40 μg/g; $n = 4$–7 per group). *C*: LPS-induced activation of NF-κB is inhibited by targeted overexpression of an IκBα suppressor mutant. Mice underwent SFO-targeted gene transfer of AdNF-κBluc with cotransfer of an IκBα phosphorylation-resistant mutant (AdS32/36A IκBα, $n = 3$) or a control vector (AdLacZ, $n = 7$) and were challenged with systemic LPS (8 μg/g ip) 30 days later. Peak luminescence signal was measured before and 4 h following LPS challenge. *$P < 0.05$ vs. AdNF-κBluc + AdS32/36AIκBα.

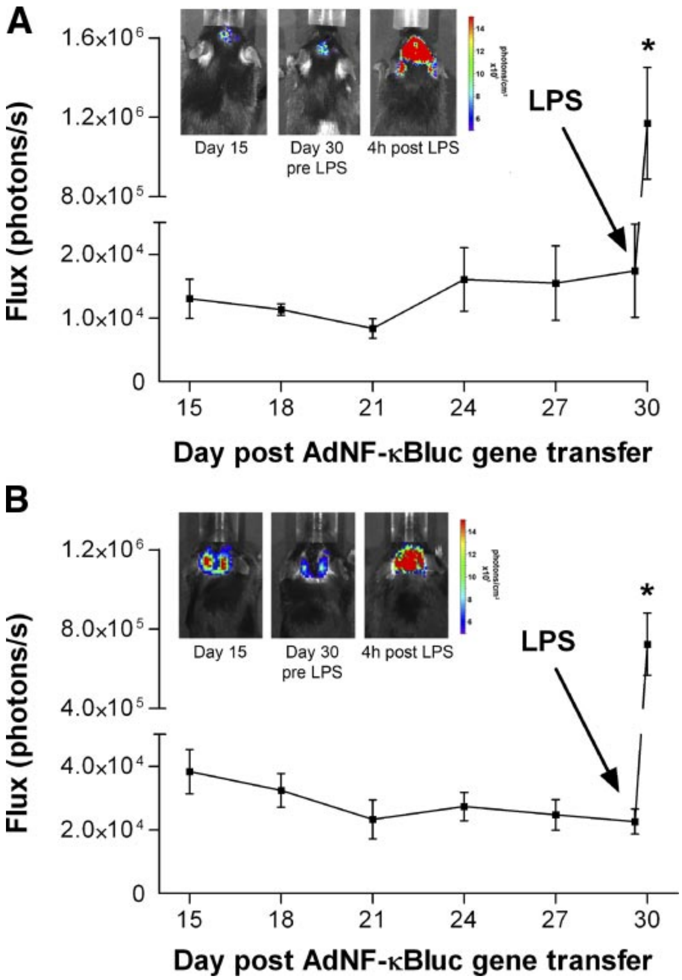

Fig. 3. NF-κB-driven luciferase expression is stable and inducible 1 mo after gene transfer to the paraventricular nucleus (PVN) or rostral ventrolateral medulla (RVLM). Bioluminescence profiles following targeted AdNF-κBluc delivery to the PVN (*A*, $n = 4$) or RVLM (*B*, $n = 6$). Peak luminescence signal was monitored every 2–4 days for 1 mo. At 30 days postgene transfer, systemic LPS challenge (8 μg/g ip) induced a dramatic increase in NF-κB-dependent luminescence in the PVN and RVLM. This dose of LPS was utilized to avoid increased mortality following higher (40 μg/g) doses of LPS observed in previous studies. *Insets*: typical bioluminescence overlays at defined time points. Areas of high photon emission are displayed as red, and areas of low photon emission are displayed as blue. $^*P < 0.05$ vs. pre-LPS.

brain slices taken through either the PVN or RVLM immediately after in vivo images were captured at 4 h post-LPS. As shown in Fig. 5, targeting of AdAP-1-luc to the PVN resulted in a robust bioluminescence signal detected in brain slices at the level of the PVN, but not at the level of the RVLM. Similarly, targeting of the AP-1-luc reporter to the RVLM resulted in a robust signal at the level of the RVLM but not the PVN. Thus, expression of the reporter construct was limited to the brain region targeted. Collectively, these results further demonstrate the utility of BLI, coupled with targeted delivery of adenovirus-mediated luciferase reporters, for longitudinal monitoring of transcription factor activation in cardiovascular regulatory nuclei.

DISCUSSION

Lasting structural and functional changes in cardiovascular regulatory circuits are likely mediated by modulations in neu-

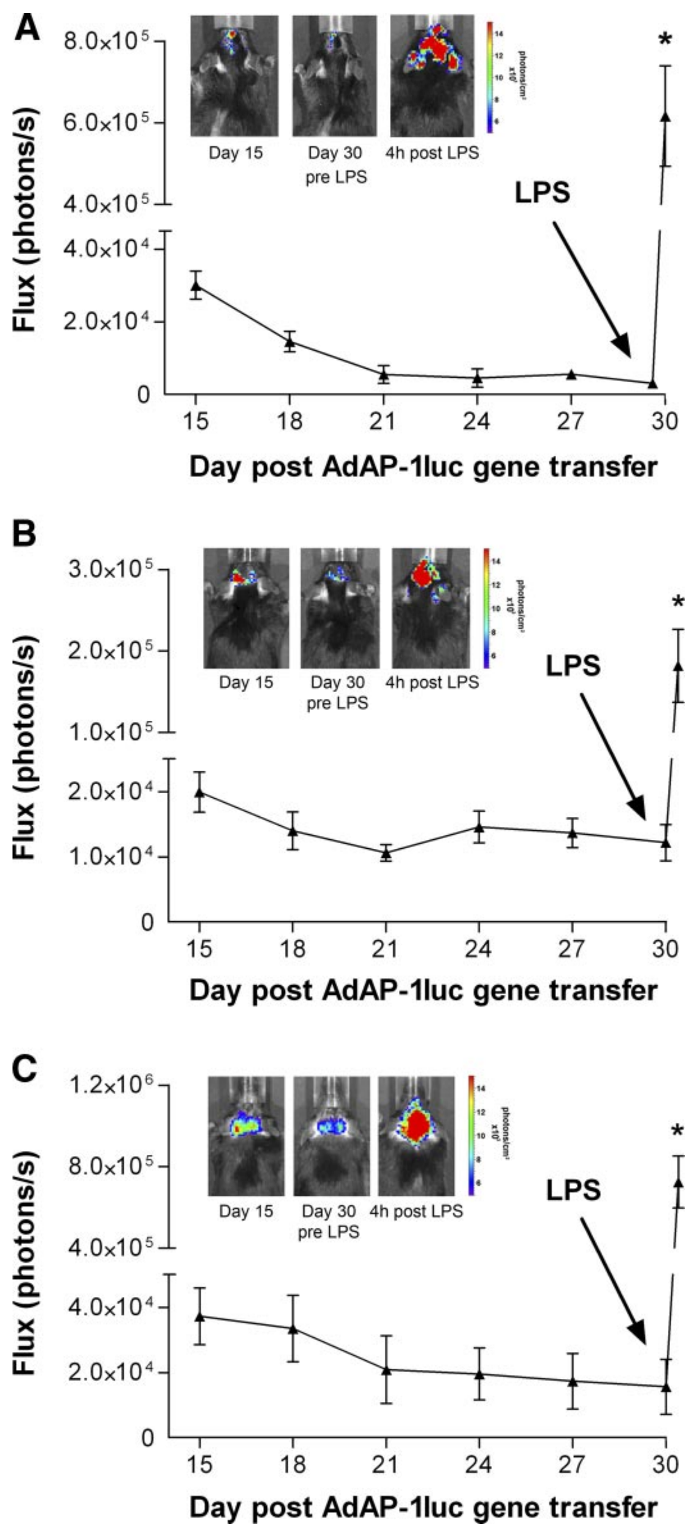

Fig. 4. Activator protein (AP)-1-driven luciferase expression is stable and inducible 1 mo after adenovirus-mediated gene transfer to central cardiovascular regulatory nuclei. Bioluminescence profiles following targeted AdAP-1luc delivery to the SFO (*A*, $n = 4$), PVN (*B*, $n = 4$), or RVLM (*C*, $n = 7$). Peak luminescence signal was monitored at every 2–4 days for 1 mo. At 30 days postgene transfer, systemic LPS challenge (8 μg/g ip) induced a robust increase in AP-1-dependent luminescence in all 3 nuclei. *Inset images*: typical bioluminescence overlays at defined time points. Areas of high photon emission are displayed as red, and areas of low photon emission are displayed as blue. $^*P < 0.05$ vs. pre-LPS.

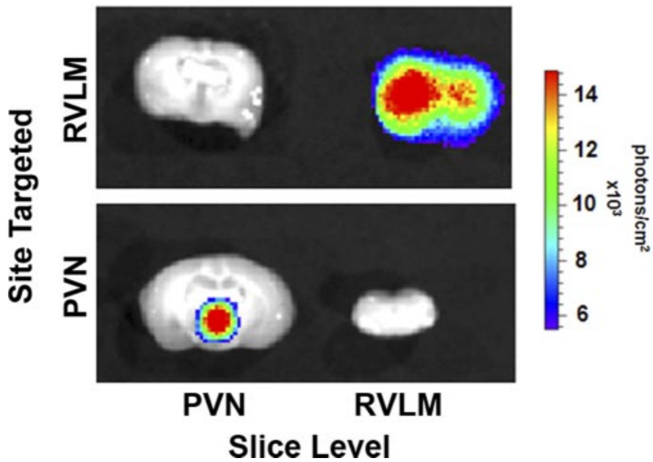

Fig. 5. AP-1-dependent luminescence is localized to brain regions targeted. One month following PVN or RVLM targeted delivery of AdAP-1luc, coronal brain sections (1 mm) were taken at the level of the PVN and RVLM 4 h following systemic LPS challenge (8 μg/g ip), and bioluminescence signal was visualized. Representative images of brain sections in which the AdAP-1-luc construct was targeted to the RVLM (*top*) or PVN (*bottom*) are shown. Areas of high photon emission are displayed as red, and areas of low photon emission are displayed as blue.

ronal gene expression patterns. Though changes in transcription factor activity within central autonomic nuclei have indeed been linked with cardiovascular disease (2, 21, 30), studies to date have been limited to select time points, primarily because current biochemical assays prohibit the creation of a complete temporal map of transcription factor activity in distinct CNS nuclei during the entire course of chronic disease. Here, we demonstrate the feasibility of in vivo BLI, in combination with adenovirus-mediated transgene delivery to the CNS, to longitudinally quantify transcription factor activation patterns in discrete cardiovascular regulatory nuclei. Following adenovirus-mediated gene transfer of NF-κB or AP-1 luciferase reporter constructs to the SFO, PVN, or RVLM, in vivo BLI allowed for daily measurements of NF-κB and AP-1 activity in these targeted brain regions in individual animals over time. While baseline activity of these transcription factors in the CNS was stable and detectable, systemic LPS challenge induced pronounced spikes in both NF-κB- and AP-1-dependent bioluminescence at 4 wk after initial gene transfer, demonstrating the ability of these reporter constructs to respond to stimuli during the course of chronic in vivo studies.

To confirm the optimal parameters for measuring bioluminescence in the brain, we first verified the kinetics and stability of the bioluminescence signal following adenoviral delivery of CMV-driven luciferase to autonomic nuclei. The observed kinetics of the bioluminescence signal in the brain following luciferin administration confirms and extends previous studies in both the periphery (11) and in the CNS (6, 16). Our studies suggest that the peak bioluminescence signal can be reliably measured in the same animal at minimum intervals of 4 h, enabling up to six measurements per day. Similarly, the sustainability of adenovirally mediated luciferase expression in the brain over months confirms previous reports by our group and others demonstrating long-lasting stability of transgene expression following adenoviral delivery to CNS nuclei (14, 35). Interestingly, Deroose et al. (16) recently reported stable lentivirus-mediated expression of luciferase in the CNS for up to 1 yr. While we have not examined the properties of adenovirus-mediated luciferase at these longer time points, the stability of luciferase expression in CNS nuclei over a 2 mo duration enables prolonged studies of reporter gene expression in CNS nuclei in vivo.

Both NF-κB and AP-1 are ubiquitous transcription factors in the CNS involved in a wide range of neurobiological functions. For example, NF-κB plays a known role in hippocampal long-term potentiation and has been implicated in Alzheimer's disease (23), while detection of the AP-1 subunit, c-fos, is often used as a marker of neuronal activation (13, 19). Of the various agonists of NF-κB and AP-1 activity, endotoxin is known to induce vigorous and widespread activation of these transcription factors in the CNS (32). Importantly, the compensatory cardiovascular responses to LPS, i.e., the release of vasopressin and ACTH, involve neural and humoral pathways that parallel those of other blood-borne stimuli involved in neuro-cardiovascular regulation, such as angiotensin II (ANG II) (3, 15, 32, 37). Thus, given the relative ease of endotoxin administration, systemic LPS challenge in this study provided a convenient and relevant model system for rapid testing of the responsiveness of NF-κB and AP-1 luciferase reporter constructs in CNS nuclei. To date, studies of LPS-induced NF-κB and AP-1 activity in the CNS have required the death of multiple cohorts of animals at numerous time points (3, 15, 32). In the present study, in vivo BLI allowed for noninvasive, real-time monitoring of activation of these transcription factors in the SFO, PVN, and RVLM at baseline conditions and in response to systemic endotoxin.

Applications of this technique offer enormous potential for long-term studies of transcription factor activation in CNS nuclei during the course of cardiovascular disease. Though studies utilizing conventional biochemical assays have implicated a potential role for both NF-κB and AP-1 in central cardiovascular regulation (21, 30), temporal maps of the activation patterns of these transcription factors in the CNS during the onset and progression of cardiovascular disease are incomplete. The majority of studies of AP-1 activity in cardiovascular regulatory nuclei have utilized c-fos immunohistochemistry to relate neuronal activation patterns with specific cardiovascular events. For example, systemic infusion of ANG II, a peptide with tremendous influence on cardiovascular homeostasis, increases c-fos staining in neurons of the lamina terminalis (26). We have shown that myocardial infarction-induced heart failure also results in increased c-fos staining in the hypothalamus (20). In addition, Liu et al. (21) recently reported a link between increased AP-1 DNA binding in the RVLM with pacing-induced heart failure in rabbits, while Chan et al. (7) demonstrated ANG II-dependent c-fos activation in the RVLM that depends on a PKCβ-NADPH oxidase-EKR1/2 cascade. While such studies hint at the potential role of AP-1 in central cardiovascular regulation, they fail to directly address the functional influence of AP-1 on transcription. Furthermore, unlike AP-1, few studies have examined NF-κB activation in central cardiovascular nuclei. However, a number of reports have demonstrated activation of this transcription factor in peripheral tissues during the pathogenesis of cardiovascular disease (4, 33). Thus, given the importance of this transcription factor in long-term neuronal processes (23), there exists a strong rationale for the study of NF-κB signaling in central cardiovascular regulatory nuclei. We are currently utilizing in vivo BLI for the study of both NF-κB and AP-1 in cardiovascular regulatory networks during the onset and progression of hypertension and heart failure.

A limitation of in vivo BLI is variability in final photon intensity due to the absorption and scattering of light through tissue (8). In fact, this may be one of the reasons for the variability seen in some of our data, e.g., Fig. 1. Photon scattering and tissue attenuation can also cause difficulties with spatial resolution; however, because we have utilized adenoviral delivery of reporter constructs to restrict luciferase expression to the discrete CNS nuclei, the source of the bioluminescence signal originates only in the brain regions that have undergone gene transfer. Thus, given the extremely low background bioluminescence of normal tissue, any photons emitted and measured at the surface of the animal have originated from targeted CNS nuclei. We have previously established our ability to restrict transgene expression to specific forebrain autonomic nuclei using adenoviral vectors (35, 36). Importantly, bioluminescence images taken of ex vivo brain slices revealed that the luminescence signal was restricted to the brain region targeted, confirming proper localization of our reporter constructs. In addition, Deroose et al. (16) have demonstrated a strong correlation between luminescence detected at the scalp with immunohistochemical analysis of luciferase expression ex vivo following lentivirus-mediated delivery of luciferase to the striatum. Thus, we are confident that in the present study the bioluminescence signal measured at the surface of the animal provides a faithful account of NF-κB- or AP-1-dependent luciferase expression in the SFO, PVN, and RVLM.

In conclusion, we have established the power and utility of in vivo BLI, coupled with adenovirus-mediated delivery of luciferase reporters, for the longitudinal mapping of transcription factor activation in CNS autonomic nuclei. This strategy provides the unique opportunity to quantify levels of functional transcription factor activity over time in the same animal and allows for the visualization of temporal patterns of transcription factor activation in discrete brain regions over extended time periods. Thus, this technique provides an innovative means of longitudinally mapping transcription factor activation patterns in targeted brain regions in vivo and has the potential to reveal functional relationships between transcription factor activation in CNS circuits with biological events in rodent models of human disease.

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