Glaucoma is a group of progressive optic neuropathies that are a leading cause of blindness worldwide. Although multiple factors, such as age, ethnicity, and family history, contribute to the risk of developing glaucoma, it is well established that sensitivity to elevated intraocular pressure (IOP) is a major cause of glaucoma and its progression. Unfortunately, IOP remains the only modifiable risk factor, although several additional strategies are in development. Clinical approaches to lower IOP include use of topical medications, laser treatment of the trabecular meshwork, or surgery. Glaucoma treatments aimed at lowering IOP are a tremendous socioeconomic burden in the United States, even to those commercially insured. Despite this investment, up to 40% to 50% of certain patient populations will still progress to irreversible vision loss, leading to significant health care challenges for the patients and their families. Thus, there is an unmet need to identify new treatment options for patients with glaucoma. With several studies showing that fluctuation of IOP is an independent risk factor for progression of glaucoma, identification of the central nervous system (CNS) pathways responsible for circadian fluctuation of IOP could provide novel targets for new glaucoma therapeutics aimed at reducing those fluctuations.

Orexin-containing neurons have been identified as playing a key role in regulating circadian behaviors, such as

**Purpose.** Intraocular pressure (IOP) remains the only modifiable risk factor for glaucoma progression. Our previous discovery that stimulation of nuclei within the hypothalamus can modulate IOP, intracranial pressure (ICP), and translaminar pressure difference (TLPD) fluctuations led us to investigate this pathway further. Our purpose was to determine the role of orexin neurons, primarily located in the dorsomedial hypothalamus (DMH) and perifornical (PeF) regions of the hypothalamus, in modulating these pressures.

**Methods.** Sprague Dawley rats were pretreated systemically with a dual orexin receptor antagonist (DORA-12) at 30 mg/Kg ($n = 8$), 10 mg/Kg ($n = 8$), or vehicle control ($n = 8$). The IOP, ICP, heart rate (HR), and mean arterial pressure (MAP) were recorded prior to and following excitation of the DMH/PeF using microinjection of the gamma-aminobutyric acid (GABA)$_A$ receptor antagonist bicuculline methiodide (BMI).

**Results.** Administration of the DORA at 30 mg/Kg significantly attenuated peak IOP by $5.2 \pm 3.6$ mm Hg ($P = 0.007$). During the peak response period (8–40 minutes), the area under the curve (AUC) for the 30 mg/Kg DORA cohort was significantly lower than the control cohort during the same period ($P = 0.04$). IOP responses for peak AUC versus DORA dose, from 0 to 30 mg/Kg, were linear ($R^2 = 0.18$, $P = 0.04$). The IOP, ICP, heart rate (HR), and mean arterial pressure (MAP) were recorded prior to and following excitation of the DMH/PeF using microinjection of the gamma-aminobutyric acid (GABA)$_A$ receptor antagonist bicuculline methiodide (BMI).

**Conclusions.** DORAs have the potential to attenuate asynchronous changes in IOP and in ICP and to lessen the extent of TLPDs that may result from central nervous system (CNS) activation.

Keywords: glaucoma, hypothalamus, orexin, intraocular pressure
feeding, wakefulness, panic response,20,21 and vigilance,22,23 and these neurons play a strong role in the regulation of neuroendocrine and autonomic functions (for a review see Ref. 24). Orexins, also known as hypocretins, are a novel class of peptide neurotransmitters discovered at the end of the 20th century.25,26 Post-translational modification of orexin transcripts results in 2 active neuropeptides; the 33-amino acid orexin A and the 28-amino acid orexin B (also known as hypocretin 1 and hypocretin 2, respectively). These neuropeptides are endogenous ligands for the G-protein-coupled receptors, orexin 1 receptor (OX1R) and orexin 2 receptor (OX2R).22,26,27 found predominately throughout the CNS but also in some peripheral neural and endocrine tissues.28–32 Accordingly, it has been shown that orexin-containing neuronal cell bodies are located almost exclusively in the nuclei of the dorsomedial hypothalamus (DMH) and in neurons of the adjoining perifornical (PeF) region of the brain.33,34 and, interestingly, also in the retina.34,35 Data support direct connectivity between gamma-aminobutyric acid (GABA)ergic neurons and orexin neurons of the DMH/PeF36–38 with strong direct and indirect projections from the suprachiasmatic nucleus (SCN) to the DMH/PeF.39–43 Thus, these neurons are ideally situated to modulate the circadian fluctuations in IOP as well as IOP fluctuations evoked by chemical stimulation of the DMH/PeF region.

Gamma-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the brain. Because many neurons in the hypothalamus, including DMH and PeF neurons, are under tonic GABA inhibition,44,45 a GABA receptor antagonist, such as bicuculline methiodide (BMI), can be microinjected into a region of interest in order to “disinhibit” neurons and cause them to fire. The resulting physiologic changes caused by this neuronal activation can then be recorded. Our previous data showed that microinjection of BMI (30 pmol/75 nL) into the DMH/PeF region caused a significant increase in heart rate (HR), mean arterial pressure (MAP), intracranial pressure (ICP), and IOP, whereas microinjection of saline vehicle had no effect on these parameters.46 The cardiovascular responses, which included the increases in HR and MAP approximately 5 to 10 minutes post-injection, were consistent with our previous findings47,48 and others.49–52 However, the discovery that chemical stimulation of the DMH/PeF region evoked increases in both ICP and IOP was completely novel. Additionally, whereas BMI caused a significant increase in HR, MAP, ICP, and IOP, the time each took to reach its peak value was not uniform.46 The peak IOP increase was significantly delayed compared to ICP resulting in a temporal phase shift along this pressure axis, which may have biomechanical implications in the optic nerve head that could lead to retinal ganglion cell degeneration and the pathogenesis of glaucoma.46

Here, we target the orexin neurotransmitter system with a dual-receptor antagonist (DORA-12) of both the OX1R and OX2R receptors to investigate the role of the orexin neurotransmitter system in regulating hypothalamically mediated increases in both the IOP and ICP, the effect of the DORA on the timing of the responses, and whether there may be coordination with cardiovascular responses.

Methods

All experiments were approved by the Institutional Animal Care and Use Committee and adhered to all standards set forth in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (240–340 g; Harlan, Indianapolis, IN, USA) were housed in pairs on a 12-hour light-dark cycle (lights on at 0700), with access to food and water ad libitum. At least 48 hours were allowed for habituation in the animal facility before any testing.

DORA Preparation and Administration

Prior to experiments, the DORA (DORA-12; Merck & Co.) was prepared for oral administration, as described and characterized previously.46,53–55 Briefly, the appropriate amount of DORA was dissolved in a 20% V/V solution of vitamin E esterified with polyethylene glycol 1000 succinate (vitamin E TPGS; Fisher Scientific) in distilled water. Five days prior to the experiments, the rats were trained with a mock gavage using control vitamin E TPGS vehicle, 0.2 mL/100 g rat weight. On the experimental day, the rats received an oral gavage of control vehicle (n = 8), 10 mg/kg (n = 8), or 30 mg/kg (n = 8) of the DORA. The DORA antagonist had demonstrated a balanced potency for OX1R and OX2R, good brain exposure, good oral bioavailability, and a favorable brain-to-plasma ratio. The 30 mg/kg PO dose of the DORA-12 used here had been shown to achieve a plasma maximum concentration (Cmax) of 2.02 μM with cerebrospinal fluid levels of 66 nM. Using brain homogenates, OX2R occupancy of the DORA-12 was analyzed ex vivo. Maximum OX2R occupancy was >90% for both 10 and 30 mg/kg doses and was demonstrated to occur 60 minutes following PO administration of the DORA-12 compound. The binding terminal half-life (t1/2) was reported as 57.5 minutes.53 The DORA-12 compound is a close structural analogue of Suvorexant.

Experimental Design

After an absorption period of 70 minutes following gavage, the animals were anesthetized with isoflurane and underwent surgery for placement of a femoral cannula for HR and MAP monitoring, a trans-cisterna-magna ventricular cannula for ICP monitoring, and craniotomy for stereotactic needle access to the hypothalamus. Preparation of the animals, surgical procedures, and cannulation methods have been previously described.46 Coordinates for craniotomy and stereotactic injection were calculated using the Paxinos and Watson atlas for the rat brain.46 Using the bregma as a reference point, the region of the DMH/PeF was targeted for microinjection (approximately 3.24 mm posterior, 0.8 mm lateral, and 8.4 mm ventral to the bregma). After the craniotomy was completed, the glass pipette was backfilled with the GABA receptor antagonist BMI (0.4 mM BMI in 0.9% normal saline with 10% v/v yellow Fluorospheres; Molecular Probes, 0.04 microns). The filled pipette was then lowered to the appropriate depth, as reported previously.46,47 Cyanoacrylate glue was used to create a watertight seal around the micropipette at the site of entry into the skull. After completion of all surgical procedures, isoflurane concentration was reduced to 1.5% and sufficient time, 10 to 30 minutes, was allowed to ensure animals had reached a steady HR, MAP, core body temperature, ICP, and IOP. After 10 minutes of stable baseline physiologic measures were recorded, the animals received a microinjection of BMI (30 pmol/75 nL) targeted to the DMH/PeF region using graded puffs of compressed nitrogen through the picoinjector to deliver the injectate. Rationale for the dose and
A schematic of coronal sections of a rat brain adapted from the atlas of Paxinos and Watson showing the injection sites for BMI in rats pretreated PO with DORA 30 mg/Kg (red), DORA 10 mg/Kg (green), or control vehicle (black). Numbers represent the anatomic level in relation to the bregma. DMN, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamus; PH, posterior hypothalamus; f, fornix; mt, mammillothalamic tract. Reprinted with permission from Paxinos G, Watson C. The Rat Brain in Stereotaxic Coordinates. 6th ed. San Diego, CA: Academic Press; 1997. Copyright 1997 Elsevier.
Injection sites within the hypothalamus were determined by the localization of FluoSpheres that were co-injected with the BMI. Injected sites were mapped (Fig. 1) and presented on anatomic representations of the rat brain.56

RESULTS

The gavage procedure using vehicle control had no significant effect on the response profiles to BMI injection for HR, MAP, ICP, or IOP as they were each similar in peak values (Table) and dynamics to the responses of animals receiving the BMI injection without prior gavage (see Samuels et al., 201246). However, a partial systemic blockade of the orexin receptors 1 and 2 with a gavage administration of the DORA (30 mg/kg) attenuated the net rise in IOP by 5.4 ± 1.3 mm Hg compared to the vehicle control animals (P = 0.007, independent t-test, 95% confidence interval [CI]; Fig. 2A).

The IOP area under the curve (AUC) for the period 8 to 40 minutes post-injection was significantly greater in the vehicle control group than for the animals receiving the 30 mg/Kg DORA (see vertically shaded region, Fig. 2A; P = 0.037; Student’s t-test). Although the IOP AUC response values from the lower gavage dose of 10 mg/Kg DORA were not significantly different than those of the control group, the IOP dose response curve, from 0 to 30 mg/Kg DORA, was linear (R² = 0.18, P = 0.04; Fig. 2B).

The baselines for ICP trended downward, although not significantly, with increasing DORA dose (Fig. 2C). Accordingly, ICP values for net rise above baseline, or for the peak AUC (0–20 minutes) were not significantly different between the control cohort and the groups of animals receiving either dose of DORA. However, relative to the control gavage animals, the raw ICP values were significantly attenuated by the 30 mg/Kg DORA administration at every time point from 6 to 12 minutes following BMI injection (see vertically shaded region, Fig. 2C, P < 0.05, 1-way ANOVA with post hoc Tukey’s HSD). The combined mean of ICP values during the peak period (4–16 minutes) showed linear dose-dependence with DORA dose (R² = 0.24, P = 0.014; Fig. 2D).

Notably, neither HR nor MAP peak responses to DMH/PeF activation were significantly affected by the DORA at 30 or 10 mg/Kg relative to control vehicle (HR = +76 ± 11, +69 ± 4, and +88 ± 8 bpm, respectively; MAP = +18 ± 2, +20 ± 2, and +24 ± 3 mm Hg, respectively).

Pretreatment of the animals with the DORA prior to chemical stimulation of the DMH/PeF region attenuated the translaminar pressure difference relative to control animals (Fig. 3A). The cumulative potential insult to the optic nerve head tissues caused by asynchronous IOP and ICP pressure increases are represented here as the AUC for each dose with the units being IOP*min (Fig. 3B). Pretreatment with both

### Table. Net Peak Increases in IOP, ICP, HR, and MAP following BMI Injection in Control Gavage Cohort Versus Published Data

<table>
<thead>
<tr>
<th>Peak Rise Above Background</th>
<th>Present Study</th>
<th>From Samuels 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>IOP</td>
<td>+10.6 ± 1.3 mm Hg</td>
<td>+7.1 ± 1.9 mm Hg</td>
</tr>
<tr>
<td>ICP</td>
<td>+4.1 ± 1.4 mm Hg</td>
<td>+3.6 ± 0.7 mm Hg</td>
</tr>
<tr>
<td>HR</td>
<td>+88 ± 12.1 bpm</td>
<td>+69 ± 8.5 bpm</td>
</tr>
<tr>
<td>MAP</td>
<td>+24 ± 3.0 mm Hg</td>
<td>+23 ± 1.6 mm Hg</td>
</tr>
</tbody>
</table>

Mean and SE are presented. None of these control data in the present study are significantly different from previously published data.46

![FIGURE 2. Systemic administration of a DORA causes a dose dependent attenuation of the increase in IOP (panels A and B) and ICP (panels C and D) following microinjection of BMI (30 pmol/75 nL) into the DMH/PeF region. All injections at t = 0 minutes (n = 8 per treatment group). *Shading A and C represents period of significant difference of vehicle versus DORA 30 mg/kg. Smoothed curves (λamda = 0.05) are presented with a 95% confidence interval. B and D Shading represents 95% confidence intervals for lines.](https:// iovs.arvojournals.org)
Chemical stimulation of the hypothalamus in the orexin-rich region of the DMH/PeF nuclei using the GABAA antagonist BMI was shown to dramatically and significantly raise the IOP, ICP, and the vasopressor parameters of HR and MAP.56,58 We and others have shown that cell bodies within the DMH/PeF project to, and synapse directly with, sympathetic relays such as the posterior raphe nuclei in the brainstem53,62 where orexin and catecholamine signaling play important roles.53–58 Further, HR and MAP stimulation resulting from chemical activation of the DMH/PeF was previously shown to project through the raphe pallidus to the sympathetic chain.57,66 Although orexin has been identified as a neurotransmitter in the connection between the DMH/PeF and presympathetic-premotor neurons,67 the administration of the DORA did not significantly attenuate the HR or MAP of the anesthetized animals in this study suggesting that vasopressor responses to hypothalamic activation are not solely orexin-mediated. However, we cannot definitely state whether or not orexin-rich neurons of the DMH/PeF projecting to the RP were solely responsible for the hypothalamically mediated IOP and ICP changes either. Understanding the established link of IOP with circadian rhythms and the suprachiasmatic nucleus,58–70 together with the evidence for orexigenic cells and neurons within the retina34,35,71–73 that project to the SCN34,35,74 and then to the DMH/PeF,39–41,75,76 it is likely that attenuation of IOP and ICP by the DORA shown here involved multiple orexigenic neural pathways. That the DMH/PeF region of the hypothalamus has a role in modulating IOP is clear, and future investigations with single orexin receptor antagonists may elucidate a more nuanced role for IOP and ICP regulation through what are more recently seen as distinct functions for the two different orexin receptors.77,78

The 30 mg/Kg PO dose of the DORA-12 used here had been previously given to unanesthetized rats in a separate study and had been reported to ameliorate panic behavior responses but not to alter the vasopressor or the thermal status of the rats, nor did it appear to sedate the animals.79 In similar fashion, the same dose of the DORA-12 in our anesthetized animals did not appear to alter the HR or MAP responses to BMI activation of the DMH/PeF relative to controls. Interestingly, in previous work, we demonstrated that the orexin neurons within the DMH were activated in anxiety responses, demonstrating greater proportions of cells showing C-Fos immunoreactivity.80 Collectively, these data would support the hypothesis that pharmacological antagonism of both the orexin receptor types in the DMH/PeF using a DORA may have an effect limited to the attenuation of ICP, IOP, and anxiety without significantly affecting cardiovascular parameters. Systemic administration of orexin receptor antagonists was used in these experiments as the efferent target nucleus or nuclei mediating the physiologic response has yet to be identified. More targeted administration may be possible in the future as the neural network is elucidated. Further, collecting useful IOP and ICP data in unanesthetized rats, although challenging, may offer new insights and should be considered in the advancement of this line of study, helping to resolve the potential links among IOP and ICP, HR, and MAP. Nonetheless, stress is seen as a covariate in glaucoma progression,81,82 and, as we have shown here, DORA administration attenuated IOP and ICP when chemically activating the DMH/PeF. Thus, a potential role for DORAs in limiting glaucoma progression

**Figure 3.** DORAs attenuate the translaminar pressure profiles in response DMH/PeF activation. Pretreatment with a DORA attenuated the translaminar pressure differences evoked by chemical stimulation of the DMH/PeF region (A) thus, hypothetically, reducing the asynchronous stress and strain at the optic nerve head. The AUC of each treatment group for the period 0 to 50 minutes (B) shows a significant difference (*) between the vehicle-administered cohort and both DORA treatment groups (1-way ANOVA followed by post hoc Tukey’s HSD). Smoothed curves (lamda = 0.05) are presented in A with a 95% confidence interval. Box plots B include median with whiskers extended to outliers.

**Discussion**

As introduced above, orexin-containing neuronal cell bodies are located almost exclusively in the hypothalamic DMH/PeF region53,55 and data supports direct connectivity between GABAAergic neurons and orexin neurons.36–39,59 It follows that, in a recently published study examining the cardiovascular vasopressor role of the orexins, the great majority of BMI-responsive sites within the DMH/PeF region that were examined were also orexin-responsive (15/18), with 12/12 sites examined within the DMH/PeF region being responsive to both orexin and BMI.59

The 30 mg/kg (P = 0.045) and 30 mg/kg (P = 0.015) dose of DORA significantly decreased the AUC for the translaminar pressure differences following chemical stimulation of DMH/PeF neurons (1-way ANOVA with post hoc Tukey’s HSD).
may extend to alleviating stress, hypertension, and other glaucoma covariates that involve central hypothalamic and orexin-mediated pathways. However, the known actions of systemic DORA administration, although transient, include day-time sleepiness, potential diminishment of motor coordination or muscle strength, or diminishment of cognition and memory. New DORA formulations that minimize these untoward effects are in development.

Importantly, our peak BMI response data obtained following the control gavage were similar to data reported previously that had been obtained without the additional handling and stimulation that a gavage procedure might induce. Baseline data, prior to BMI injection, in the control gavage-treated group (data not shown) were not greater than what we have previously reported either. A gavage training session for each subject in the week prior to the experiments may have enabled this consistency between results from differing experimental designs.

The dose-response to the DORA administration is evident from the charts presented in Figures 2A and 2C, whereas the linear nature of a dose-response plot for both IOP and ICP is supportive (see Figs. 2B, 2D). Although the ICP data can be inherently variable and did not support a dose-response using AUC data, as did the IOP response, the raw ICP values, nonetheless, depicted a significant dose-response for ICP. It was not clear why the baseline ICP values for the 30, 10, and 0 mg/Kg DORA gavage were not more consistent with each other, although the mean baseline differences were not significant. It appeared, however, that pre-dosing with the DORA may have slightly lowered the baseline ICP, and, if substantiated in future investigation, the long-term implications on trans-laminar pressure differences and glaucoma progression would be of interest and clinically feasible given that the DORA-12 compound used here is an orexin receptor antagonist like the DORA compound Suvorexant recently approved by the US Food and Drug Administration (FDA) and commercialized for the treatment of insomnia (for a review, see Ref. 85).

Chemical stimulation of different locations in or near the same nuclei of the hypothalamus can respond differently to an identical stimulus. Figure 1 shows that, although all of the 24 BMI injection sites were in the region of the DMH/PeF, there was injection site variability within groups and between groups. It cannot be ruled out, therefore, that the conclusions drawn from these data about the dose-dependent attenuation by the DORA on IOP and ICP may be merely a function of injection site variability. Future studies aimed at controlling the chemical stimulus by reducing injectate dose and volume and site variability will help resolve this. Further, orexin receptor knockout rats, although not yet available, would provide an alternative approach in determining the role of the orexin neurotransmitter system in IOP and/or ICP regulation, and this approach will be pursued.

As axons of the optic nerve leave the back of the eye, they pass through the lamina cribrosa, a complex biomechanical structure composed of a three-dimensional framework of flexible connective tissue beams, which normally protect retinal ganglion cell axons as they enter the retrobulbar space. In doing so, neurons pass from a relatively high-pressure environment within the eye to a lower pressure cranial compartment. It is the alternating bidirectional stress and strain on the lamina cribrosa over time, potentially related to asynchronous fluctuations in ICP and IOP, that has been implicated in the glaucomatous pathophysiology occurring at the lamina cribrosa and the optic nerve in this region, followed by the loss of vision that is associated with glaucoma. Our data have shown that, although BMI activation of the DMH/PeF caused a significant increase in HR, MAP, ICP, and IOP, the time that each took to reach its peak value was not synchronous. The peak IOP increase was significantly delayed compared to ICP (28 vs. 6 minutes post-injection, respectively), resulting in a temporal phase shift that created a trans-optic nerve head pressure range that was much larger than the changes in IOP or ICP alone. This finding is critical from a biomechanical standpoint because it suggests that the stresses and strains on the optic nerve head are likely larger than we originally predicted based on changes in IOP or ICP alone. In addition, the dynamics of the DORA-attenuated IOP and ICP responses were similarly asynchronous to the IOP and ICP responses obtained without DORA pretreatment, as previously reported, suggesting that the DORA attenuation did not substantially alter the neural pathways utilized by the chemical hypothalamic stimulation.

The asynchrony of the ICP and IOP responses to chemical stimulation of the DMH/PeF would predict that a dose-dependent attenuation of both IOP and ICP to DORA administration would result in an attenuation of the overall translaminar pressure profile by the DORA. Indeed, the AUC for both the 30 mg/Kg and the 10 mg/Kg PO dose of the DORA likely ameliorated the stress/strain effect of an asynchronous IOP and ICP stimulation caused by the BMI microinjection into the DMH/PeF. This result has important ramifications for the advancement of orexin-targeted pharmaceutical development for the management of glaucoma, but also demonstrates how clinically significant trans-laminar pressure differences induced by a single hypothalamic activation event might be minimized. Given that there are significant direct and indirect projections from the suprachiasmatic nucleus to orexinergic neurons in the DMH/PeF region, this pathway merits further study to determine its role in circadian fluctuation of IOP and determine its potential as a target for novel glaucoma therapeutics.

We conclude that a dual orexin receptor antagonist administered PO has the potential to ameliorate the asynchronous changes in IOP and in ICP and to lessen the extent of translaminar pressure differences that may result from CNS activation. Novel approaches to arresting vision loss in glaucoma are needed, and investigating the regulation of IOP and ICP through orexin-mediated strategies is warranted.

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Disclosure: A.A. DeCarlo, None; N. Hammes, None; P.L. Johnson, Merck (F); A. Shekhar, Merck (F); B.C. Samuels, Merck (F)

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