Characterization of genetic differences within the centrally projecting Edinger–Westphal nucleus of C57BL/6J and DBA/2J mice by expression profiling

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INTRODUCTION

The Edinger–Westphal (EW) nucleus is a compact region that extends along the rostral–caudal axis of the midline within the ventromedial periaqueductal gray of the midbrain. While this region has been historically described as a cholinergic population of preganglionic neurons projecting to the ciliary ganglion to control oculomotor functions, detailed examinations have revealed that the EW is comprised of two distinct nuclei. One population of EW preganglionic (EWpg) neurons was found to control oculomotor functions, and a separate population of EW centrally projecting (EWcp) neurons was found to contain stress- and feeding-related neuropeptides. Although it has been shown that EWcp neurons are highly responsive to drugs of abuse and behavioral stress, a genetic characterization of the EWcp was needed. To identify genetic differences in the EWcp of inbred mouse strains that differ in behaviors relevant to EWcp function, we used publicly available tools from the Allen Brain Atlas to identify 68 transcripts that were selectively expressed in the EWcp, and examined their expression within tissue punch microdissection samples containing the EWcp of adult male C57BL/6J (B6) and DBA/2J (D2) mice. Using 96-well quantitative real-time PCR (qPCR) arrays that included the EWcp-specific genes, several other genes of interest, and five housekeeping genes, we identified strain differences in expression of 11 EWcp-specific genes (B6 vs. D2 mice, and several of these were verified either at the protein level using immunohistochemistry (IHC) or in silico using microarray data sets from whole brain and other brain areas. These results demonstrate a significant advance in our understanding of the EWcp on three levels. First, we generated a list of EWcp-specific genes (most of which had not yet been reported within the EWcp in the literature) that will be informative for future studies of EWcp function. Second, due to similarity in results from qPCR and IHC, we revealed that strain differences in basal EWcp neuropeptide content are accounted for by differential transcription and number of peptidergic neurons, rather than by differential rates of peptide release. And third, our identification of differentially expressed EWcp-specific genes between B6 and D2 mice may hold powerful insight into the neurogenetic contributions of the EWcp to stress- and addiction-related behaviors.

Keywords: Edinger–Westphal, midbrain, oculomotor, neuropeptide, inducible transcription factor, immediate early gene, alcohol, urocortin

Detailed examination of the midbrain Edinger–Westphal (EW) nucleus revealed the existence of two distinct nuclei. One population of EW preganglionic (EWpg) neurons was found to control oculomotor functions, and a separate population of EW centrally projecting (EWcp) neurons was found to contain stress- and feeding-related neuropeptides. Although it has been shown that EWcp neurons are highly responsive to drugs of abuse and behavioral stress, a genetic characterization of the EWcp was needed. To identify genetic differences in the EWcp of inbred mouse strains that differ in behaviors relevant to EWcp function, we used publicly available tools from the Allen Brain Atlas to identify 68 transcripts that were selectively expressed in the EWcp, and examined their expression within tissue punch microdissection samples containing the EWcp of adult male C57BL/6J (B6) and DBA/2J (D2) mice. Using 96-well quantitative real-time PCR (qPCR) arrays that included the EWcp-specific genes, several other genes of interest, and five housekeeping genes, we identified strain differences in expression of 11 EWcp-specific genes (B6 vs. D2 mice, and several of these were verified either at the protein level using immunohistochemistry (IHC) or in silico using microarray data sets from whole brain and other brain areas. These results demonstrate a significant advance in our understanding of the EWcp on three levels. First, we generated a list of EWcp-specific genes (most of which had not yet been reported within the EWcp in the literature) that will be informative for future studies of EWcp function. Second, due to similarity in results from qPCR and IHC, we revealed that strain differences in basal EWcp neuropeptide content are accounted for by differential transcription and number of peptidergic neurons, rather than by differential rates of peptide release. And third, our identification of differentially expressed EWcp-specific genes between B6 and D2 mice may hold powerful insight into the neurogenetic contributions of the EWcp to stress- and addiction-related behaviors.

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Sterrenburg et al., 2011), as well as stimuli related to food restriction (Xu et al., 2009, 2011). Finally, the existence of two distinct nuclei was also indicated by the finding that neuropeptide-containing neurons of the EWcp projected primarily to limbic brain areas, rather than to the ciliary ganglion (Loewy and Saper, 1978; Loewy et al., 1978; Bittencourt et al., 1999; Bachtell et al., 2004; Weitemier and Ryabinin, 2005a).

Thus, the EWcp emerged as a recently identified (and therefore, poorly characterized) brain region that appeared to be especially important for regulation of responses to addictive drugs and environmental challenges (Ryabinin and Weitemier, 2006; Koizic, 2007; Koizic et al., 2011). In particular, our interest in this nucleus originated from several neural mapping studies in which the EWcp consistently showed a selective induction of the inducible transcription factor (ITF) c-Fos (Fos) following oral self-administration of alcohol (Toppole et al., 1998; Bachtell et al., 1999, 2003; Ryabinin et al., 2001, 2003; Weitemier et al., 2004). Further experiments revealed that alcohol-induced neural activity within the EWcp occurred in 95−100% of neurons containing the neuropeptide urocortin-1 (Ucn1; Bachtell et al., 2002b; Ryabinin et al., 2003; Spangler et al., 2009). These findings suggested a potential role for EWcp–Ucn1 neurons in alcohol-related phenotypes, a hypothesis that was confirmed by studies showing that electrolytic lesions encompassing the EWcp dramatically attenuated EtOH preference in C57BL/6J (B6) mice (Bachtell et al., 2004). However, these results were obtained in B6 mice that exhibited greater Ucn1-IR in individual EWcp neurons, more numerous (and larger in size) than those of DBA/2J (D2) mice (Bachtell et al., 2002b). However, these results were obtained using a Thionin stain, which did not allow differentiation between Ucn1 neurons of the EWcp, cholinergic neurons of the EWpg, and dopaminergic (DAergic) neurons of the adjacent rostral linear nucleus of the raphé (RL). An additional study found that B6 mice exhibited greater Ucn1-IR in individual EWcp neurons, relative to D2 mice (Weitemier and Ryabinin, 2005a). These data raised the possibility that, in addition to being driven in part by differences in the total number of neurons, the observed strain differences in EWcp–Ucn1 protein expression could also be due to differences in Ucn1 mRNA expression, or Ucn1 peptide release.

In order to determine whether differences in peptide expression could be attributed to differential expression at the mRNA level, we performed tissue punch microdissection of the EWcp region and quantified the expression of several EWcp-selective genes between B6 and D2 mice. These two well-characterized strains differ in alcohol-, stress-, and feeding-related phenotypes, which might be reflective of genetic differences within the EWcp (Ryabinin et al., 1999; Lewis et al., 2007; Yoneyama et al., 2008). Several transcripts that appeared to be selectively expressed within the EWcp were identified by resources present in the Allen Brain Atlas (ABA1; Lein et al., 2007). We extracted RNA from micropunches containing the EWcp and subjected samples to customized, 96-well plates that allowed quantitative real-time PCR (qPCR) analysis of these transcripts.

After identifying several genes that were differentially expressed within the EWcp of B6 and D2 mice by qPCR array, we used immunohistochemical (IHC) and in situ analyses to verify some of these findings. In doing so, we demonstrate that strain differences at the protein level are unlikely to be attributed to differential rates of peptide release, and are more likely due to differences in gene transcription and cell number. As such, the present findings identify several previously unexplored genes that may be integral for addiction- and stress-related behaviors regulated via the mammalian EWcp.

**MATERIALS AND METHODS**

**ANIMALS**

We studied adult (8- to 10-week-old) male C57BL/6J (B6) and DBA/2J (D2) mice that had been delivered from The Jackson Laboratory (Sacramento, CA, USA) and housed four per cage in our colony. Information from the Jax Phenome Database lists mean body/brain weights (in grams) for 8-week-old male B6 and D2 mice as 24.3/0.423 and 22.8/0.354, respectively. All mice received ad libitum access to food (LabDiet 5001; Richmond, IN, USA) and water, and were maintained on a 12-h light–dark schedule (lights on at 06:00 hours). All experiments were performed with strict adherence to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**IDENTIFICATION OF EWcp-SPECIFIC TRANSCRIPTS**

We used resources available from the ABA to identify transcripts that appeared to be selectively expressed within the EWcp. The initial goal of the ABA project was to perform *in situ* hybridization using probes targeted against every protein-coding gene in the mouse genome in order to visualize patterns of expression within the brains of adult male B6 mice. The ABA group has systematically documented the brain expression of several 1000 mRNA transcripts, and have made this information publicly available online (Allen Brain Atlas, 2004; Lein et al., 2007).

Important for our study, the ABA has developed a “fine structure” search feature that allows searching for genes that are expressed in smaller brain structures. We began our search by browsing the expression patterns of the 50 genes identified by the fine structure feature as being located within the “Edinger–Westphal.” However, because the spatial resolution of this search feature is relatively low and does not represent the vast coronal span of the EWcp, we verified that only 27 of these 50 transcripts appeared to be selectively expressed within the EWcp. Reasons for...
exclusion of the other genes included either a pattern of expression that was not within the EWcp, a non-specific pattern of expression that included the EWcp as well as several other structures, or the appearance of very low expression within the EWcp.

Next, we used the "neuroblast" feature on the ABA website to find genes with similar expression patterns to those identified by the initial fine structure search. This allowed a rapid method for discovering additional genes that were also selectively expressed within EWcp. Finally, we used the AGEA gene finder, another ABA tool that finds genes within a specific brain area by allowing the user to choose any voxel in the mouse brain as a seed region and then identifying genes with expression patterns that are highly correlated with that seed space. By placing seed regions in five different voxels throughout the mouse midbrain (centered around the EWcp), we were able to identify additional genes that were specifically expressed within the EWcp, yet had not been identified by prior methods.

Thus, after beginning with 7–10 candidate transcripts that we had known were EWcp-specific (based on our prior studies and on literature searches of the EW nucleus), we were able to identify a total of 68 genes that appeared to be selectively expressed within the EWcp. It is important to note that our analysis, which relied heavily on the features included on the ABA website, was prone to false negatives (i.e., in situ probe failure). Therefore, rather than being a liberal method for assembling a list of EWcp-specific genes, this list is likely an underestimate of the number of EWcp-specific genes that are highly expressed in the adult mouse brain. The 68 identified genes were further interrogated by the qPCR array approach, as described below.

**ADDITIONAL TRANSCRIPTS OF INTEREST**

Additional transcripts that were not selectively expressed in the EWcp were also included in the analysis, and were comprised of the following five groups: (1) three immediate early genes encoding inducible transcription factors (ITFs), included to assess differences in basal activity between strains because they are well-established markers of neuronal activity; (2) eight genes related to the dopamine (DA) system, included because the tissue punch information approach. Furthermore, a few of the identified genes were also analyzed at the protein level by IHC, and all identified genes were analyzed in silico using microarray data, providing additional confirmation.

**IN SILICO ANALYSES**

Following identification of genes exhibiting strain differences in EWcp expression, we used GeneNetwork3 (GeneNetwork, 2001; Chesler et al., 2004) as an additional resource for verifying expression differences. Analysis of several microarray data sets determined whether the transcripts showing genotype-dependent expression within EWcp also differed in expression throughout whole brain, cerebellum, striatum, hippocampus, hypothalamus, neocortex, and amygdala.

For each of the identified genes, we compared the reported values for B6 and D2 mice from the following GeneNetwork data...
Table 1 | Complete list of all genes of interest included in the analysis.

| Gene name        | Entrez ID | Category          | Gene name        | Entrez ID | Category          |
|------------------|-----------|-------------------|------------------|-----------|-------------------|
| A730017C20Rik    | 225583    | EWcp-specific     | Prmt2            | 15468     | EWcp-specific     |
| Adcyap1          | 11516     | EWcp-specific     | Psme1            | 19186     | EWcp-specific     |
| Arhgdig          | 14570     | EWcp-specific     | Psme2            | 19188     | EWcp-specific     |
| Art10            | 56796     | EWcp-specific     | Ptpm              | 19275     | EWcp-specific     |
| BC023892         | 212943    | EWcp-specific     | Rbp4             | 19662     | EWcp-specific     |
| Brunol6          | 76183     | EWcp-specific     | Rcn1             | 19672     | EWcp-specific     |
| Btg3             | 12228     | EWcp-specific     | Rps12            | 20042     | EWcp-specific     |
| Bves             | 23828     | EWcp-specific     | Rps5             | 20103     | EWcp-specific     |
| C530008M17Rik    | 320827    | EWcp-specific     | Rgs4             | 19736     | EWcp-specific     |
| Cart              | 27220     | EWcp-specific     | Scg2             | 20254     | EWcp-specific     |
| Cck               | 12424     | EWcp-specific     | Sidr1            | 320007    | EWcp-specific     |
| Cds2             | 110911    | EWcp-specific     | Sicc39a6         | 106957    | EWcp-specific     |
| Cpeb1             | 12877     | EWcp-specific     | Sncg             | 20618     | EWcp-specific     |
| Chrc1             | 68588     | EWcp-specific     | Sirt2            | 20733     | EWcp-specific     |
| Ctxn1             | 330695    | EWcp-specific     | Ssr1             | 107513    | EWcp-specific     |
| Dlk1              | 13386     | EWcp-specific     | Syt5             | 53420     | EWcp-specific     |
| Dnajc12          | 30045     | EWcp-specific     | Tacr2            | 21337     | EWcp-specific     |
| Erp29            | 67397     | EWcp-specific     | Tmed3            | 66111     | EWcp-specific     |
| Fxyd8            | 59095     | EWcp-specific     | Tmem22           | 245020    | EWcp-specific     |
| Gabre             | 14404     | EWcp-specific     | Tppp3            | 67971     | EWcp-specific     |
| Gap43             | 14432     | EWcp-specific     | Tpc6             | 22068     | EWcp-specific     |
| Ghar              | 208188    | EWcp-specific     | Ucn              | 22226     | EWcp-specific     |
| Gpx3             | 14788     | EWcp-specific     | Vat1             | 26949     | EWcp-specific     |
| Hap1             | 15114     | EWcp-specific     | Zchc12           | 72693     | EWcp-specific     |
| Itgb1            | 16412     | EWcp-specific     | Egr1             | 13653     | ITFs              |
| Klh1             | 93688     | EWcp-specific     | Fos              | 14281     | ITFs              |
| Mlec             | 109154    | EWcp-specific     | Fosb             | 14282     | ITFs              |
| Mrap2             | 244958    | EWcp-specific     | Th                | 21823     | DA-related        |
| Ly6h             | 23934     | EWcp-specific     | Ddc               | 13195     | DA-related        |
| Mesdc2           | 67943     | EWcp-specific     | Slc6a3           | 13162     | DA-related        |
| Ndn               | 17984     | EWcp-specific     | Drd1a            | 13488     | DA-related        |
| Nef               | 66208     | EWcp-specific     | Drd2             | 13489     | DA-related        |
| Neto1            | 246317    | EWcp-specific     | Drd3             | 13490     | DA-related        |
| Npc2             | 67963     | EWcp-specific     | Drd4             | 13491     | DA-related        |
| Nucb2            | 53322     | EWcp-specific     | Drd5             | 13492     | DA-related        |
| Pcdn16           | 245578    | EWcp-specific     | Ntsr1            | 18216     | VTA-related       |
| Pck1             | 18548     | EWcp-specific     | Chrna5           | 110835    | VTA-related       |
| Peg10            | 170676    | EWcp-specific     | Chrna6           | 11440     | VTA-related       |
| Peg3             | 18616     | EWcp-specific     | Chrnbp3          | 108043    | VTA-related       |
| Pgr15l           | 245526    | EWcp-specific     | Chhr1            | 12921     | CRF-related       |
| Pid3             | 18807     | EWcp-specific     | Chhr2            | 12922     | CRF-related       |
| Postn            | 50706     | EWcp-specific     | Chrbp            | 12919     | CRF-related       |

In addition to five housekeeping genes (see Table 2) and five wells dedicated to genomic DNA-, RT-, and PCR-controls, the 96-well qPCR array included 68 EWcp-specific genes, three ITFs, eight DA-related genes, four VTA-related genes, and three CRF-related genes.

sets: UCHSC BXD Whole Brain M430 2.0 (Nov06) RMA, SJUT Cerebellum mRNA M430 (Mar05) RMA, HQF BXD Striatum ILM6.1 (Dec10v2) Ranklnv, Hippocampus Consortium M430v2 (June06) PDNN, INIA Hypothalamus Affy MoGene 1.0 ST (Nov10), HQF BXD Neocortex ILM6.1 (Dec10v2) Ranklnv Database, and INIA Amygdala Cohort Affy MoGene 1.0 ST (Mar11) RMA (Saba et al., 2006; Overall et al., 2009). Data are presented as mean ± SEM, and significance threshold was set at p < 0.05. All significant findings identified by t-test are detailed in Table 4.

IMMUNOHISTOCHEMICAL ANALYSES

Immunohistochemistry was performed on products of three genes identified as being differentially expressed between B6 and D2 mice by the qPCR array. The selection of gene products was based
Table 2 | Cycle thresholds (CTs) for the five housekeeping genes included on the array.

| Gene name | Entrez ID | C57BL/6J | DBA/2J | t-Value | p-Value |
|-----------|-----------|-----------|--------|---------|---------|
| Actb      | 11461     | 20.31 (±0.068) | 20.56 (±0.119) | 1.584 | 0.1442 |
| Gapdh     | 14433     | 19.15 (±0.104) | 19.56 (±0.066) | 3.493 | 0.0058 |
| Gusb      | 110006    | 28.66 (±0.127) | 28.58 (±0.136) | 0.415 | 0.6888 |
| Hprt1     | 15452     | 21.71 (±0.084) | 21.50 (±0.178) | 0.913 | 0.3828 |
| Hsp90ab1  | 15516     | 19.86 (±0.058) | 19.95 (±0.170) | 0.444 | 0.6665 |

Only Gapdh was found to have CTs that differed between strains (p < 0.01). Thus, Gapdh was excluded from the list of housekeepers used to quantify the genes of interest.

For each gene product, six to eight slices containing the EWcp (evenly spaced along the rostral–caudal axis, from −3.2 to −3.8 mm from bregma) were chosen from each animal. Examination of CCK- and Ptprn-IR were preceded by an antigen retrieval process. However, antigen retrieval was not necessary for examination of CART-IR, which stains heavily within mouse EWcp neurons even without this additional step (Kozicz, 2003; Cservenka et al., 2010).

For IHC procedures examining CCK and Ptprn in the EWcp, antigen retrieval consisted of rinsing the sections in PBS and then boiling the tissue in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) followed by cooling to room temperature. For all IHC procedures, slices underwent a standard DAB staining protocol identical to previous reports from our lab (Spangler et al., 2009; Giardino et al., 2011a), with the exception that primary antibodies were directed against either human CART (55–102 (H-003-60, Phoenix) human/rat/mouse CCK 26–33 (H-069-04, Phoenix), or human Ptprn (HPA-007179, Sigma-Aldrich), and were used at concentrations of 1:20,000, 1:30,000, and 1:1000, respectively. Dehydration and coverslipping methods were also identical to previous reports.

The number of CART-, CCK-, or Ptprn-positive neurons within the EWcp was counted manually using a Leica DM4000 microscope and recorded by an observer. A single value per animal was calculated by averaging the cell counts across all slices from that subject, and mean cell counts for the two strains were compared by t-test separately for each of the three gene products. One data point was excluded from the analysis of CART-IR in B6 mice, because the value was greater than 2.5 SD below the mean. No other outliers were identified. Data are presented as mean ± SEM, and significance threshold was set at p < 0.05.

RESULTS

ANALYSIS OF HOUSEKEEPING GENES

Preliminary analysis of the five housekeeping genes included on the qPCR arrays revealed that Gapdh CT values were significantly greater in D2 vs. B6 mice (t10 = 3.49; p < 0.01; Table 2). CT values for other housekeeping genes were not different between strains (all t10 < 1.59; all p > 0.14). When Gapdh CT values were normalized to the average CT values of the remaining four housekeeps by the 2^-ΔCT method, analysis revealed that Gapdh expression was greater in B6 vs. D2 mice (t10 = 2.71; p < 0.05). Therefore, all genes of interest included on the array were normalized to the average of the remaining four housekeeping genes (Actb, Gusb, Hprt1, Hsp90ab1), with Gapdh excluded.

on available commercial antibodies. Ucn1 and Fos were two gene products that were not included in these analyses because our previous studies had already identified differences in Ucn1-IR and Fos-IR between B6 and D2 mice (Bachtell et al., 2002b; Weitemier et al., 2005).

After habituation to our mouse colony, mice (n = 8 per strain) were euthanized by CO2 and underwent transcardial perfusion with 2% PFA dissolved in H2O. Brains were rapidly dissected and placed in 2% PFA for storage overnight, followed by cryoprotection in 20 and 30% sucrose dissolved in phosphate buffered saline (PBS) containing 0.1% NaN3. Coronal sections were sliced 30 μm thick on a Leica CM1850 cryostat, and slices were collected in PBS containing 0.1% NaN3.

For IHC procedures examining CCK and Ptprn in the EWcp, antigen retrieval consisted of rinsing the sections in PBS and then boiling the tissue in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) followed by cooling to room temperature. For all IHC procedures, slices underwent a standard DAB staining protocol identical to previous reports from our lab (Spangler et al., 2009; Giardino et al., 2011a), with the exception that primary antibodies were directed against either human CART (55–102 (H-003-60, Phoenix) human/rat/mouse CCK 26–33 (H-069-04, Phoenix), or human Ptprn (HPA-007179, Sigma-Aldrich), and were used at concentrations of 1:20,000, 1:30,000, and 1:1000, respectively. Dehydration and coverslipping methods were also identical to previous reports.

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GENE EXPRESSION ANALYSES

After normalization to the four remaining housekeeping genes, 14/86 genes of interest were found to differ significantly in expression between B6 and D2 strains: BC023892 (also known as Fam46a), Btg3, Bves, Cart, Cck, Ghst, Nett1, Postn, Pitpn, Rcn1, Ucn, Egr1 (also known as zif268), Fos, and Drd5 (Figure 2). In each case, expression was greater in B6 mice, relative to D2 mice (all \( t_{10} > 2.44; p < 0.05 \); Table 3).

Of these 14 genes, 11 were selectively expressed and/or enriched within the EWcp (Figures 3 and 4). Two of these genes were ITFs (Egr1 and Fos), which are not selectively expressed within EWcp, but have been known to be induced within EWcp following certain environmental stimuli (Bachtell et al., 1999; Ryabinin et al., 2001). The remaining gene, Drd5 (which encodes the dopamine receptor subtype 5) is not known to be selectively expressed within EWcp, but was included with the smaller list of DA-related genes.

IN SILICO ANALYSES

Of the 14 transcripts demonstrating strain differences in EWcp expression, six of these (Btg3, Bves, Cart, Cck, Egr1, and Rcn1) were confirmed to also have significant differences in expression within whole brain and/or other brain regions (cerebellum, striatum, hippocampus, hypothalamus, neocortex, amygdala; Table 4). Consistent with qPCR array results from EWcp micropunches, all gene expression levels were greater in B6 vs. D2 mice, with the exception of Rcn1, whose genotype-dependent regulation in whole brain, cerebellum, and amygdala appeared to be opposite from that in the EWcp (Table 4).

IMMUNOHISTOCHEMICAL ANALYSES

In order to determine whether gene expression differences could be replicated at the protein level, we developed IHC staining protocols to visualize neurons immunoreactive for either CART, CCK, or Pitpn within the EWcp. In each case, we identified a greater number of immunoreactive neurons in B6 mice, relative to D2 mice (all \( t_{13-14} > 5.08; p < 0.0005 \); Figure 5), consistent with the results from analyses of Cart, Cck, and Pitpn in the qPCR array.

DISCUSSION

The current study took advantage of publicly available tools in the ABA to identify several genes that were selectively expressed within the EWcp, and used tissue punch microdissection of the EWcp in combination with array expression profiling to quantify those transcripts (along with several other genes of interest) within tissue samples of the EWcp from adult male B6 and D2 mice. Our results, which expand on several previous studies that analyzed protein-level expression of ITFs and neuropeptides within the EWcp, confirm that the mRNA levels of several EWcp-specific genes and two ITFs are greater within B6 mice, relative to D2 mice. Interestingly, these findings are paralleled by differences in alcohol-related phenotypes among alcohol-prefering B6 mice and alcohol-avoiding D2 mice (Crabbe et al., 1983; Cunningham et al., 1992; Yoneyama et al., 2008).

Although prior evidence based on EWcp lesions and EWcp–Ucn1 protein expression in alcohol-prefering vs. alcohol-avoiding rodent strains suggested that EWcp–Ucn1 neurons promote alcohol drinking and food consumption (Bachtell et al., 2004; Weitemier and Ryabinin, 2005b), additional studies using intracranial injections showed that Ucn1 also decreased alcohol drinking and food consumption (Spina et al., 1996; Ryabinin et al., 2008). Thus, both a decrease in EWcp–Ucn1 tone (via. EWcp lesions) and an increase in Ucn1 tone (via. intracranial Ucn1 infusions) had similar effects on these two behaviors. One potential explanation for this apparent contradiction could be that higher Ucn1-IR within the EWcp of alcohol-prefering vs. alcohol-avoiding animals (including B6 vs. D2 mice) resulted from lower neuronal activity and less release of Ucn1 from the EWcp, rather than greater levels of Ucn1 mRNA.

Our current data provide a strong argument against a lower rate of release in B6 mice, because levels of Ucn1 mRNA were higher in the EWcp of these animals, mimicking the differences in protein expression. Thus, differences in Ucn1-IR within the EWcp of B6 vs. D2 mice are likely attributed to higher levels of Ucn1 mRNA within individual neurons (as well as a difference in the number of EWcp–Ucn1 neurons), rather than lower neural activity and lower rates of peptide release. Because EWcp–Ucn1 protein levels are reflective of EWcp–Ucn1 mRNA levels, these data support our longstanding hypothesis that greater activity of Ucn1 neurons...
within the EWcp is associated with a genetic predisposition toward greater alcohol intake and heightened alcohol sensitivity (Bachtell et al., 2003; Ryabinin and Weitemier, 2006). This hypothesis is also supported by our recent study in which genetic deletion of Ucn1 blunted alcohol preference and alcohol reward in mice on a B6 background (Giardino et al., 2011b).

In addition, levels of Fos and Egr1 mRNA were greater in the EWcp of B6 vs. D2 mice, arguing against the possibility that greater Ucn1-IR in B6 vs. D2 mice was due to less Ucn1 release. Although we did not directly compare baseline levels of Fos-IR in the current study, a previous experiment found that the number of Fos-IR cells was greater in B6 vs. D2 mice (Bachtell et al., 2003), consistent with our gene expression data. Since Fos and Egr1 are well-characterized markers of neural activity, this suggests that basal activity of the EWcp is higher in B6 vs. D2 mice. Given this presumed difference in neural activity, peptide release from the EWcp is likely to be higher in B6 vs. D2 mice, rather than vice versa.

An additional possibility for the seemingly contradictory relationship between Ucn1 tone and alcohol-related phenotypes was that lesions of the EWcp had the potential to eliminate DA neurons of the RLi, which intermingle with EWcp–Ucn1 neurons (Bachtell et al., 2002a; Gaszner and Kozicz, 2003; Fonareva et al., 2009). However, because there are more DA-synthesizing neurons in the RLi of alcohol-avoiding D2 mice as compared to alcohol-prefering B6 mice (D’Este et al., 2007), it remains unclear whether this neuronal population could contribute to alcohol intake and reward. Interestingly, despite this difference in the number of RLi neurons, no significant differences in transcripts characteristic of D2ergic neurons were detected by qPCR.

We expanded our earlier studies examining Ucn1-IR and Fos-IR in the EWcp of B6 and D2 by detecting significantly more neurons immunoreactive for CART, CCK, and Ptprn in B6 vs. D2 mice. The protein product of Cart (cocaine- and amphetamine-regulated transcript) is a neuropeptide important for mediating drug reward and regulating food intake (Rogge et al., 2008). Our IHC analyses showed that CART has an extremely dense pattern of expression within the EWcp, a finding previously demonstrated by our lab and others across several mammalian species (Koylu et al., 1998; Kozicz, 2003; Lima et al., 2008; Cservenka et al., 2010). Here we show for the first time that EWcp–CART is differentially expressed between B6 and D2 mice at the mRNA and protein levels, suggesting that CART could be involved in similar functions as Ucn1. Since CART has been shown to colocalize with Ucn1 in EWcp (Kozicz, 2003; Cservenka et al., 2010), this result could also be due to either differences in mRNA levels per neuron and/or number of EWcp neurons between B6 and D2 mice.

The protein product of Cck (cholecystokinin) is a neuuropeptide important for several functions, including regulation of food intake, anxiety-like behavior, and drug reward (Beglinger, 2002; Rotzinger and Vaccarino, 2003). Although the presence of CCK in the mammalian EWcp has been demonstrated previously (Maciewicz et al., 1984; Rattray et al., 1992), this is the first time that CCK peptide has been reported in the mouse EWcp. It is tempting to speculate that EWcp–CCK is involved in similar functions as EWcp–Ucn1 and EWcp–CART.

Although we were unable to generate a suitable IHC procedure for the protein product of growth hormone secretagogue receptor (Ghsr; the receptor for the orexigenic hormone ghrelin), previous studies from our laboratory implicate EWcp–Ghsr involvement in a mouse model of binge-like alcohol consumption (Kaur and Ryabinin, 2010), consistent with our finding of greater Ghsr mRNA expression in B6 vs. D2 mice.

Ptprn encodes protein tyrosine phosphatase, receptor type N (also known as islet antigen 2; IA-2). Other than the ABA, we are the first to report that this gene is expressed in the mammalian

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Table 3 | Genes of interest showing expression differences between strains by qPCR array of EWcp micropunch.

| Gene Name | C57BL/6J | DBA/2J | t-Value | p-Value | Effect | ABA Link |
|-----------|----------|--------|---------|---------|--------|---------|
| BC023892  | 0.0568 (+0.0067) | 0.0224 (+0.0018) | 5.720 | 0.0002 | B6 > D2 | tinyurl.com/BC023892 |
| Btg3      | 0.1036 (+0.0130) | 0.0723 (+0.0048) | 2.565 | 0.0281 | B6 > D2 | tinyurl.com/Btg3ABA |
| Bves      | 0.0052 (+0.0018) | 0.0016 (+0.0002) | 2.440 | 0.0349 | B6 > D2 | tinyurl.com/BvesABA |
| Cart      | 7.278 (+1.427) | 1.765 (+0.3247) | 4.330 | 0.0013 | B6 > D2 | tinyurl.com/CartABA |
| Cck       | 1.415 (+0.2723) | 0.4343 (+0.0564) | 4.167 | 0.0019 | B6 > D2 | tinyurl.com/CckABA |
| Ghsr      | 0.0434 (+0.0031) | 0.0180 (+0.0023) | 6.688 | <0.0001 | B6 > D2 | tinyurl.com/GhsrABA |
| Neto1     | 0.0268 (+0.0029) | 0.0151 (+0.0017) | 3.712 | 0.0040 | B6 > D2 | tinyurl.com/Neto1ABA |
| Postn     | 0.1213 (+0.0142) | 0.0439 (+0.0091) | 4.821 | 0.0009 | B6 > D2 | tinyurl.com/PostnABA |
| Ptprn     | 1.671 (+0.2285) | 0.9939 (+0.1880) | 2.301 | 0.0442 | B6 > D2 | tinyurl.com/PtprnABA |
| Rcn1      | 0.0130 (+0.0015) | 0.0010 (+0.0003) | 8.581 | <0.0001 | B6 > D2 | tinyurl.com/Rcn1ABA |
| Ucn       | 4.274 (+0.8318) | 0.9971 (+0.1631) | 4.576 | 0.0010 | B6 > D2 | tinyurl.com/UcnABA |
| Egr1      | 0.0492 (+0.0078) | 0.0251 (+0.0030) | 3.263 | 0.0085 | B6 > D2 | tinyurl.com/Egr1ABA |
| Fos       | 0.0624 (+0.0147) | 0.0169 (+0.0018) | 3.676 | 0.0043 | B6 > D2 | tinyurl.com/FosABA |
| Drd5      | 0.0058 (+0.0007) | 0.0030 (+0.0005) | 3.316 | 0.0078 | B6 > D2 | tinyurl.com/Drd5ABA |
| Dcc       | 0.1138 (+0.0245) | 0.5077 (+0.1465) | 2.227 | 0.0501 | D2 > B6 | tinyurl.com/DccDABA |

Values are mean arbitrary units (2-ΔCT), with SEM in parentheses. All expression differences were in the direction of B6 > D2, with the exception of Ddc (p = 0.0501), which demonstrated a marginally significant increase in expression within D2 mice, relative to B6 mice. Right-hand column provides link to gene expression patterns on Allen Brain Atlas.
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FIGURE 3 | Genes within the EWcp-specific category that were identified as being differentially expressed between B6 and D2 mice are indeed EWcp-specific. Shown are coronal slices at approx. −3.5 mm from bregma from adult male B6 mouse brains that have undergone in situ hybridization to reveal the EWcp-specific expression of BC023892 (A), Btg3 (C), Bves (E), Cart (G), Cck (I), Neto1 (K), and Rcn1 (M). The close-up images (B,D,F,J,L,N) show the area within the dotted line of the corresponding figure, indicating that BC023892, Btg3, Bves, Cart, Cck, Neto1, and Rcn1 demonstrate an EWcp-specific pattern of expression. Scalebar = 100 μm, and is valid for all close-up images. Images courtesy of the Allen Brain Atlas, used with permission.

FIGURE 4 | Genes within the EWcp-specific category that were identified as being differentially expressed between B6 and D2 mice are indeed EWcp-specific. Shown are sagittal slices at the midline from adult male B6 mouse brains that have undergone in situ hybridization to reveal the EWcp-specific expression of Ghsr (A), Postn (C), Ptpn (E), and Ucn (G). The close-up images (B,D,F,H) show the area within the dotted line, indicating that Ghsr, Postn, Ptpn, and Ucn demonstrate an EWcp-specific pattern of expression. Each scalebar = 500 μm. Images courtesy of the Allen Brain Atlas, used with permission.

EWcp. The function of this gene is not well understood, despite the fact that it is a major auto-antigen in insulin-dependent diabetes mellitus and could be involved in mediating dense core vesicle release (Lu et al., 1996; Cai et al., 2004). As such, Ptpn could
be involved in release of vesicles from the EWcp. This function, together with our identification of greater Ptprn expression in B6 vs. D2 mice, is an additional piece of evidence suggesting that EWcp neuronal activity is greater in B6 vs. D2 mice.

Use of in silico analyses as an additional confirmation of results from the EWcp qPCR array was largely successful, showing that at least six of the 14 identified transcripts also showed genotype-dependent expression throughout whole brain and/or cerebellum, striatum, hippocampus, hypothalamus, neocortex, and amygdala (Table 4). We speculate that although these transcripts display a typical EWcp-specific pattern within the midbrain, strain differences in expression of Btg3 and possibly Cck may generalize to several brain areas. On the other hand, the absence of consistent genotype-dependent expression of BC023892, Bves, Cart, Ghsr, Neto1, Postn, Ptprn, Ucn, Egr1, Fos, and Drd5 (and the opposite direction of difference for Ren1) within several analyzed brain areas strengthens our conclusion that strain differences in stress-, feeding-, and addiction-related behavior may be related to expression of these genes specifically within the EWcp.

While some of these expression differences could theoretically be confirmed by Western blotting, the difficulties of dissecting relatively large quantities of EWcp from the mouse brain prevented this analysis. We anticipate that the other transcripts expressed higher in B6 vs. D2 mice also have corresponding differences in protein levels. In fact, this would be expected to be the case for nearly all EWcp-specific proteins that are co-expressed with Ucn1, because there are more Ucn1-positive neurons in B6 vs. D2 mice. Therefore, our studies are rather conservative in confirming the selectivity of gene expression within the EWcp.

It follows that greater mRNA expression within a micropunch from the EWcp region of B6 vs. D2 mice is, by itself, suggestive evidence that the gene is selectively expressed in EWcp. Thus, our finding that expression of the DA-related gene Drd5 is greater in EWcp microdissections from B6 vs. D2 mice suggests that this transcript might be expressed in EWcp neurons. The evidence for this possibility is further strengthened by the fact that other DA- and VTA-related genes were not differentially expressed between B6 vs. D2 mice, indicating a unique pattern of expression for Drd5. Drd5 is probably the least-studied DA receptor, and its potential

### Table 4 | Confirmation of qPCR array results by in silico analyses of whole brain and brain region-specific microarray data.

| Gene name | Region       | C57BL/6J (t-Value) | DBA/2J (t-Value) | t-Value | p-Value | Effect       |
|-----------|--------------|--------------------|------------------|---------|---------|--------------|
| Btg3      | Whole brain  | 10.223 (±0.019)    | 9.987 (±0.018)   | 9.017   | <0.0001 | B6 > D2      |
| Btg3      | Cerebellum   | 9.552 (±0.049)     | 9.231 (±0.076)   | 3.550   | 0.0238  | B6 > D2      |
| Btg3      | Striatum     | 6.724 (±0.037)     | 6.547 (±0.016)   | 4.391   | 0.0482  | B6 > D2      |
| Bves      | Cerebellum   | 8.848 (±0.054)     | 8.644 (±0.048)   | 2.824   | 0.0477  | B6 > D2      |
| Cart      | Hippocampus  | 7.539 (±0.203)     | 6.666 (±0.101)   | 3.202   | 0.0493  | B6 > D2      |
| Cck       | Hypothalamus | 9.323 (±0.091)     | 9.040 (±0.037)   | 2.881   | 0.0164  | B6 > D2      |
| Cck       | Neocortex    | 15.227 (±0.020)    | 15.008 (±0.039)  | 4.997   | 0.0378  | B6 > D2      |
| Egr1      | Amygdala     | 10.707 (±0.049)    | 10.490 (±0.071)  | 2.515   | 0.0456  | B6 > D2      |
| Ren1      | Whole brain  | 9.042 (±0.066)     | 9.551 (±0.053)   | 6.013   | 0.0001  | D2 > B6      |
| Ren1      | Cerebellum   | 5.606 (±0.065)     | 6.035 (±0.111)   | 3.304   | 0.0298  | D2 > B6      |
| Ren1      | Amygdala     | 9.769 (±0.018)     | 9.988 (±0.042)   | 4.793   | 0.0030  | D2 > B6      |

Values retrieved from publicly available database sets on www.genenetwork.org (see Materials and Methods).

**FIGURE 5 | Gene expression differences in Cart, Cck, and Ptprn are verified at the protein level.** Representative images of neurons immunoreactive for (C) CART, (F) CCK, and (I) Ptprn were found within B6 mice, relative to D2 mice. Scalebar = 500 μm, valid for all representative images. **p = 0.0002; ***p < 0.0001.
expression and function in EWcp is an intriguing hypothesis that awaits further testing.

Our conservative use of two housekeeping genes to control for loading artifacts makes us confident in gene expression differences identified in the study. In that respect, it is interesting that we found that Gapdh was differentially expressed between B6 and D2 mice. Other studies have found that Gapdh can be regulated in the EWcp by stress (Dersk et al., 2008). We would hypothesize that the observed differences in EWcp Gapdh expression are reliable, because other studies have not identified differential expression of Gapdh in whole-brain analysis of B6 and D2 mice (GeneNetwork, 2001; Shirley et al., 2004). Gapdh catalyzes an important energy-yielding step in carbohydrate metabolism, which could also serve as an indication of higher activity in the EWcp of B6 vs. D2 mice.

Taken together, we have identified at least 11 transcripts that are preferentially expressed in the EWcp, and differentially present in the EWcp of B6 vs. D2 mice. Further examination of these transcripts could shed light on the function of this recently characterized brain region, and could provide insight into the genetic underpinnings of behavioral differences between B6 and D2 mice, which serve as models of contrasting behavioral phenotypes (including susceptibility to alcoholism, addiction, stress, and anxiety).

In broader terms, our approach illustrates how a combination of data-mining and genetic techniques can overcome the technical difficulties inherent in analyzing a distinct neuronal population. For example, the tissue punch samples that we used for our analyses contained a region larger than the EWcp itself, and the search features on the ABA provided fairly low spatial resolution. However, we were conservative in our identification of EWcp-specific genes, which led to successful utilization of the micropunch and expression profiling methods. The combination of standard gene expression analysis with a simple bioinformatics approach may prove to be a powerful technique for advancing the field of behavioral neurogenetics.

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