EPHB6 controls catecholamine biosynthesis by up-regulating tyrosine hydroxylase transcription in adrenal gland chromaffin cells

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Running title: EPHB6 controls catecholamine biosynthesis

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Key words: Erythropoietin-producing hepatocellular receptor B6 (EPHB6), adrenal gland chromaffin cells, catecholamine, tyrosine hydroxylase, early growth response 1 (EGR1), c-JUN, cell signaling, androgen receptor, ion channel

ABSTRACT

EPHB6 is a member of the erythropoietin-producing hepatocellular kinase (EPH) family and a receptor tyrosine kinase with a dead kinase domain. It is involved in blood pressure regulation and adrenal gland catecholamine (CAT) secretion, but several facets of EPHB6-mediated CAT regulation are unclear. In this study, using biochemical, RT-qPCR, immunoblotting, and gene microarray assays, we found that EPHB6 up-regulates CAT biosynthesis in adrenal gland chromaffin cells (AGCCs). We observed that epinephrine content is reduced in the AGCCs from male Ephb6-KO mice, caused by decreased expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in CAT biosynthesis. We demonstrate that the signaling pathway from EPHB6 to TH expression in AGCCs involves Rac family small GTPase 1 (RAC1), MAP kinase kinase 7 (MKK7), C-Jun N-terminal kinase (JNK), proto-oncogene C-Jun (c-JUN), activator protein 1 (AP1), and early growth response 1 (EGR1). On the other hand, signaling via extracellular signal-regulated kinase (ERK1/2), p38 mitogen-activated protein kinase, and ELK1, ETS transcription factor (ELK1) was not affected by the EPHB6 deletion. We further report that EPHB6’s effect on AGCCs was via reverse signaling through ephrin B1, and EPHB6 acted in concert with the non-genomic effect of testosterone to control CAT biosynthesis. Our findings elucidate the mechanisms by which EPHB6 modulates CAT biosynthesis and identify potential therapeutic targets for diseases such as hypertension caused by dysfunctional CAT biosynthesis.

EPHB6 is a member of erythropoietin-producing hepatocellular kinases (EPH), the largest family of receptor tyrosine kinases (1,2). The ligands of EPH are called ephrins (EFNs), which are also membrane proteins. EFNs can trigger EPH signaling by canonic forward signaling, i.e., from ligand EFNs to receptor EPHs. However, EFNs can also receive signaling from EPHs and transduce signals into cells, and such noncanonical action (i.e., from EPHs to EFNs) is called reverse signaling (2). The interaction between EPHs and EFNs are promiscuous: one EPH can bind to multiple EFNs, and one EFN, to multiple EPHs. In general, the EPHA family...
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members bind to EFNAs, and EPHB family members binds to EFNBs (2).

EPHS/EFNs function in many organs and systems (2). We first reported the critical involvement of EPHs and EFNs in the immune system (3-15). In the past 5 years, we have demonstrated that EPHs/EFNs are involved in regulating blood pressure (BP), which was previously unknown, in a series of publications (16-25). We reported, using gene knockout mouse models, that while EPHB6, EFNB1, EFNB3 and EPHA4 deletion results in BP elevation (16,17,20,25), EPHB4 and EFNB2 deletion lowers it (18,19). Thus, members of EPHs/EFNs are a novel yin and yang system that fine-tunes BP homeostasis. In all such cases, sex hormones act in concert with these EPHs/EFNs for controlling BP. Some of these findings from the mouse model have been corroborated by human genetic studies, in which we revealed that some variants in the EFNB2, EFNB3 and EPHA4 genes or a related signaling molecule gene are significantly associated with hypertension in a sex-specific way (19,21,22, 24,25).

EPHB6 is highly expressed in the medullae of adrenal glands, which are the major source of catecholamine (CAT) in the circulation. The ambient blood CAT level reflects this hormone’s effect on the homeostasis of BP (26,27). We showed that male Ephb6 gene knockout (KO) mice produce reduced amounts of 24-h urinary CAT (16), but such phenotype disappears after castration (16). We further demonstrated that CAT release in male KO adrenal gland chromaffin cells (AGCCs) is decreased as a consequence of compromised Ca$^{2+}$ influx triggered by acetylcholine (ACh) (23). This decrease was caused by EPHB6 deletion in combination with the non-genomic effect of testosterone, and hence after castration or in females, the CAT secretion by KO AGCCs is normal (16). We further demonstrated that reduced Ca$^{2+}$ influx in male KO AGCCs is the result of augmented Big Potassium channel (BK) current, which causes an earlier closure of voltage-gated calcium channels, leading to decreased Ca$^{2+}$ influx (23).

In the present study, we report that EPHB6 also plays a critical role in regulating CAT biosynthesis. The signaling pathway from EPHB6 to CAT biosynthesis was investigated.

RESULTS

Adrenal glands from male Ephb6 KO mice showed reduced epinephrine content

Our previous study demonstrated that male but not female Ephb6 KO mice have reduced CAT secretion (16). Secretion and biosynthesis of CAT are distinct but yet interrelated events. To assess whether the KO adrenal glands were also compromised in CAT biosynthesis, we measured their CAT content, using epinephrine as a representative CAT, as our previous study showed that the levels of three major types of CAT (epinephrine, norepinephrine and dopamine) in the 24-h urine were similarly reduced in the male KO mice. As shown in Figure 1A, the epinephrine content in male KO but not female KO adrenal glands was significantly reduced, but castration brought the level of male KO AGCCs to that seen in uncastrated WT counterparts. This pattern is consistent with that of 24-hour urine CAT levels in the KO mice before and after castration (16). The size of adrenal gland medullae from male, female and castrated male KO mice was similar to their WT counterparts (Fig. 1B), suggesting that the change in the epinephrine content in the male KO mouse adrenal glands is not due to altered size of the gland medullae.

We then investigated whether the reduced epinephrine content in the male KO adrenal glands was due to the decreased levels of TH, the rate-limiting enzyme in CAT biosynthesis. As shown in Figure 1C, the TH mRNA levels were decreased in the male but not female KO glands, compared to their WT counterparts. Such a decrease was abolished after castration.

This pattern of TH expression was also confirmed at the protein level (Fig. 1D). Therefore, EPHB6 deletion correlated to reduced TH expression in a male sex hormone-dependent fashion, TH being the rate-limiting enzyme of CAT biosynthesis.

Ephb6 KO decreased the transcription factor Egr1 expression in adrenal gland medullae

To elucidate molecular mechanisms underlying the defective TH expression in the adrenal gland
medullae, we subjected the WT and KO medullae to transcriptome microarray analysis.

The array data were deposited to the Gene Expression Omnibus with accession number GSE120400.

Since the TH expression was decreased in male but not female KO medullae, and such a change disappeared after castration, we sought out for genes whose expression pattern fitted to this pattern, i.e., they were altered in male but not female KO medullae, and such alteration in males would disappear after castration. We first compared the gene expression profile of the male WT versus KO adrenal gland medullae. Twenty genes had more than 2-fold higher expression in the WT medullae than in the KO medullae, but none had more than 2-fold lower expression in such comparison, as shown in the heat map of Figure 2A (left column). The expression of these 20 genes in female WT versus KO medullae and in castrated WT versus castrated KO medullae was then compared (Fig. 2, middle and right column). Only 2 genes (LOC666403 and CFD) still showed more than 2-fold higher expression in the female WT and castrated WT medullae, compared to their KO counterparts. The remaining 18 genes resembled the expression pattern which we were looking, and could potentially be involved in regulating TH expression.

We selected the top 4 hits from the 18 genes for further investigation. The selection criterion was that they had more than 3-fold higher expression in the male WT medullae compared to KO ones (i.e., Egr1, 11.1 fold; JunB, 5.8 fold; Fos, 3.8 fold; and Dusp1, 3.2 fold). The first 3 are transcription factors and the last one is a phosphatase. Although Hspa1a had a similar high level change (3.5 fold), due to the ubiquitous presence of this heat shock protein and its being neither a transcription factor nor an enzyme, it was excluded from further study.

Even though we could confirm by RT-qPCR that the mRNA expression of JUNB, FOS and DUSP1 was significantly reduced in the male KO medullae, their protein levels showed no change (data not shown). Therefore, no further investigation was carried out on these 3 genes.

We confirmed by RT-qPCR that Egr1 mRNA expression was significantly lower in male but not in female KO medullae, compared to their WT counterparts (Fig. 2B). This decrease was ameliorated after castration. This was verified at the protein level by immunoblotting (Fig. 2C), i.e., the male but not female KO medullae had lower EGR1 protein level, and this was reversed to normal levels after castration.

Reduced EGR1 expression in KO chromaffin cells was correlated to decreased TH expression

EGR1 is a transcription factor. To prove that reduced EGR1 level was responsible for the decreased TH expression and hence reduced CAT biosynthesis, we knocked down EGR1 expression by siRNA in a non-malignant mouse AGCC cell line (tsAM5NE cells) (28). The efficiency of Egr1/EGR1 knockdown at the mRNA and protein levels was confirmed by RT-qPCR and immunoblotting (Fig. 3A). As a consequence of EGR1 knockdown, TH expression at the mRNA and protein level was reduced (Fig. 3B), indicating the EGR1 transcription factor is indeed responsible for enhancing TH mRNA transcription. It is to be noted that tsAM5NE cells, although being normal AGCCs, only produce norepinephrine but not epinephrine (28), and therefore, the former was used as a surrogate representative of CAT in our assay. The norepinephrine content in the tsAM5NE cells was decreased, as expected (Fig. 3C), after EGR1 knockdown, as a consequence of reduced TH level. Thus, these results showed that reduced EGR1 expression is correlated to reduced CAT synthesis in AGCCs from Ephb6 KO-mice.

Ephb6 KO leads to reduced AP-1 association with its binding site in the Egr1 gene enhancer

One possible mechanism by which EPHB6 may control Egr1 expression is that it acts through transcription factors, which associate with Egr1 gene enhancers. There is an AP-1 binding site between positions +383 and +393 in the 5’ untranslated region of the Egr1 gene. AP-1 promotes gene transcription when bound to enhancers, i.e., AP-1 binding sites, of many genes (29). ACh and nicotine are known to increase TH expression and consequently CAT synthesis in AGCCs. These two molecules similarly bind to nicotinic and muscarinic acetylcholine receptors.
(nAChR and mAChR, respectively). We used nicotine in lieu of ACh in this and the rest of the experiments to stimulate AGCCs as nicotine is more stable for storage and in the culture medium. According to electrophoretic mobility shift assays (EMSA), AP-1 binding to the AP-1-binding sequence in the 5′ upstream region of the Egr1 gene was reduced with the nuclear extracts of AGCCs from male Ephb6 KO mice, compared to those from WT counterparts (Fig. 4A). We confirmed that the shifted AP-1 bands in EMSA contained both c-JUN and c-FOS (Supporting Information Figs. 1A and 1B). AP-1 is a dimer of JUN and FOS family members, and c-JUN is the major subtype of JUN proteins. Nicotine-triggered c-JUN phosphorylation was compromised in male Ephb6 KO AGCCs, compared to that in the WT counterparts (Fig. 4B).

Compromised signaling pathways upstream of c-JUN in male Ephb6 KO AGCCs

We assessed the activation status of signaling molecules upstream of c-JUN. Results indicated reduced JNK phosphorylation at T173/Y185 (Fig. 5A) and also MKK7 phosphorylation at S227/T275 (Fig. 5B) in male KO AGCCs stimulated by nicotine. In these cells, G-LISA assay revealed reduced activation of the further upstream signaling molecule, RAC1 (Fig. 5C).

Using inhibitors for JNK, MKK7 and RAC1, and employing Egr1 mRNA expression as a readout, we revealed that the suppression of these signaling molecules could indeed repress Egr1 expression in AGCCs from WT male mice (Fig. 5D-F, left panels), demonstrating the relevance of the reduced activity of these molecules in male KO AGCCs to decreased Egr1 expression. On the other hand, these inhibitors had no effect on mRNA expression of an unrelated molecule Cbl (Fig. 5D-F, right panels), indicating that the effect of these inhibitors on Egr1 expression was not due to general toxicity.

On the other hand, the activation of ELK1, ERK1/2 and p38MAPK, which could theoretically promote Egr1 expression, was not changed in male KO AGCCs, compared to their WT counterparts (Fig. 6A-C).

**EPH/EFN signaling direction and the role of testosterone**

We assessed the signaling direction between EPHB6 and EFNs, by using solid phase anti-EPHB6-Ab (for forward signaling) and EPHB6-Fc (for reverse signaling). As shown in Fig. 7A, when tsAM5NE chromaffin cells were cultured on EPHB6-Fc- but not anti-EPHB6 Ab-coated wells, their nicotine-stimulated TH level was augmented, suggesting that reverse signaling from EPHB6 to EFNs enhances TH synthesis.

EPHB6 can interact with EFNB1, EFNB2 and EFNB3. We investigated which one(s) was essential for the reverse signaling. Since EFNB3 KO in mice manifested no CAT secretion phenotype (20), we focused on EFNB1 and EFNB2. As shown in Fig. 7B, solid phase anti-EFNB1 Ab but not anti-EFNB2 Ab augmented nicotine-stimulated TH expression in tsAM5NE chromaffin cells (Fig. 7B), suggesting EPHB6’s effect on CAT synthesis is mainly via EFNB1 reverse signaling.

To clarify the role of testosterone in reducing CAT synthesis in AGCCs from male KO mice, we cultured AGCCs from female KO mice in the presence or absence of testosterone. As shown in Fig. 7C, cell membrane impenetrable BSA-conjugated testosterone caused lower CAT content in AGCCs from KO but not WT female mice. This indicates that the non-genomic effect of testosterone, but not the simple presence of testicles in the adult life or during fetal development is responsible for the observed diminished AGCC CAT biosynthesis in male KO mice.

**DISCUSSION**

Our previous study shows that EPHB6 is highly expressed in the medullae of adrenal gland, and Ephb6 KO leads to reduced 24-urine CAT levels (16). We further demonstrated that EPHB6 is critical in regulating AGCC ion channel opening and consequently in controlling acute CAT secretion (23). The acute secretion of CAT from AGCCs is from stored immediately-releasable pool of CAT-containing vesicles docked near the cell membrane (30). Long-term secretion of CAT, caused by repeated stimulation of AGCCs acetylcholine, involves recruitment of vesicles...
from the reserve pool (31). The 24-h urine CAT levels reflect the sum of both acute and long-term CAT secretion. CAT biosynthesis might affect both of these processes by controlling their respective pool sizes. In order to assess this, we examined the CAT content of adrenal glands from the KO mice. Even though the size of the glands and their medullae from male and female KO mice was similar to their WT and the castrated male KO counterparts, the epinephrine content of the adrenal gland medulla from male KO mice was significantly lower compared to the WT counterparts. This suggests that CAT biosynthesis and/or degradation in the male KO adrenal gland is abnormal.

TH is the rate-limiting enzyme in CAT biosynthesis (32). The reduced TH levels in KO AGCCs likely led to the lowered CAT biosynthesis in these KO cells. TH activity in AGCCs is regulated by phosphorylation of its serine residues and by its protein level. Its S31 phosphorylation augments its activity, while S40 and S19 phosphorylation alleviates feedback inhibition (33). Examination of TH phosphorylation at S31, S40 and S19 in the adrenal glands from male KO mice without or with nicotine stimulation, revealed no consistent differences from the WT counterparts (data not elaborated). However, the TH protein and mRNA levels were reduced in the male KO adrenal glands, indicating reduced transcription from TH gene.

To elucidate the mechanisms by which EPHB6 regulates TH gene transcription, we profiled transcriptome of adrenal glands from male, female and castrated KO and WT mice. We have confirmed by RT-qPCR and immunoblotting that the expression of the top hit Egr1/EGR1 was decreased in the adrenal gland medulla of male KO mice and such a change disappeared after castration, agreeing well with the phenotype of TH expression and CAT content of the KO adrenal glands. EGR1 is a zinc-finger protein belonging to the EGR family transcription factors (34). It activates the genes containing EGR1-binding sites. We searched a 300-bp 5’ upstream sequence of the mouse (C57BL/6) TH gene with a transcription factor-finding program AliBaba2.1(35), and found 2 EGR1-binding sites at -41/-50, -112/-121 (the first nucleotide before the TH gene is designated as position -1) (Fig. 8). siRNA knockdown of EGR1 in AGCCs led to reduced TH expression at both mRNA and protein levels, with concurrent reduction of norepinephrine content in the AGCCs, proving that EGR1 indeed positively regulates TH expression. In support of our conclusion, Papanikolau et al. reported that in the 5’ upstream sequence of rat TH gene from positions -122 to -114, there is an EGR1-binding site CACCCCCGC, which is proven to positively regulate TH transcription in a reporter assay (34). This rat EGR1-binding sequence is identical to another EGR1-binding site in the mouse TH gene between positions -103 and -95 (CACCCCCGC). Our current EGR1 expression and knockdown results established the correlation between EGR1 expression levels and CAT biosynthesis.

The reduced Egr1 mRNA levels in KO AGCCs suggested that EPHB6 deletion decreased Egr1 transcription. In the 5’ untranslated region of the first exon of mouse Egr1 gene, there is an AP-1 binding site (ACTGACCTAGA) between positions +383 and +393 (the first nucleotide in exon 1 is designated as position +1; the start codon is at position +1667) (Fig. 8). This site has been proven to be functional in enhancing Egr1 transcription in a reporter gene assay in human HEK293 cells (36). Using EMSA, we found that in the Ephb6 KO AGCCs, AP-1-binding site in the Egr1 gene had less association with AP-1 transcription factor, compared to WT AGCCs. Our data established the correlation between active AP-1 binding and Egr1 expression. Earlier literature showed that the reduced association of nuclear AP-1 with its binding of its target genes is correlated to the level of its target gene transcription (37,38). There are other AP-1 binding sites in the mouse Egr1 gene 5’ sequence (positions -153 to -144) and TH gene 5’ sequence (positions -285 to -276). Conceivably, enhancer activity of all these AP-1-binding sites might be similarly affected by the reduced AP-1 association in the KO AGCCs, leading to diminished expression of Egr1 and TH transcription sequentially or simultaneously.

AP-1 is a dimeric transcription factor composed of JUN and FOS family members. JUN proteins can form homodimers or form heterodimers with FOS proteins, both of which can associate with AP-1-
EFNB1/RAC1/MKK7/JNK/JUN/AP1/EGR1/TH/CAT is suggested.

The above postulated pathway was active when the AGCCs were stimulated with AChR agonist nicotine, which binds to nAChR and mAChR, similar to acetylcholine. The major consequence of nAChR and mAChR activation by nicotine or ACh is the change of cytosolic cation concentration. This leads to larger Ca\textsuperscript{2+} influx through voltage-gated calcium channels (VGCCs). The increased Ca\textsuperscript{2+} concentration induces a cascade of downstream signaling events. These events include the activation RAC1/MKK7/JNK/JUN/AP1, as demonstrated in our experiments using WT AGCCs (Figs. 4 and 5). The details of the signaling between Ca\textsuperscript{2+} and RAC1 remain to be further elucidated. Obviously, the EFN1 reverse signaling pathway overlaps with the AChR signaling pathway starting from RAC1 in the RAC1/MKK7/JNK/JUN/AP1 cascade (Fig 8), and is needed for the optimal function or the latter as the deletion of EPHB6 (the stimulator of EFN1) caused compromised strength of this RAC1/MKK7/JNK/JUN/AP1 pathway, leading to decreased CAT synthesis.

We conducted an additional experiment to show that the non-genomic effect of testosterone was responsible for the suppressed CAT synthesis in KO AGCCs. In this experiment, AGCCs from...
female KO mice were treated with testosterone. The use of female mice guaranteed a lack of exposure of AGCCs to high levels of testosterone in vivo. While AGCCs from female KO mice in the absence of exogenous testosterone had no phenotype with regard to Egr1 expression, the testosterone treatment rendered them a phenotype similar to that seen in male KO AGCCs. This suggests that the presence of testosterone in the adult life but not during fetal development is responsible for its action in concert with EPHB6 in leading to the Egr1 phenotype, and consequently the CAT phenotype.

Our previous publication (23) suggests that such the non-genomic effect of testosterone is responsible for promoting outward K\(^+\) efflux, hence earlier closure of VGCCs. We hypothesize that under a normal circumstance, EFN1B1 has a suppressive impact on testosterone’s K\(^+\) efflux-promoting effect, as illustrated in Figure 8. In the absence of EFN1B1, such suppressive effect is released. This results in a larger K\(^+\) efflux, and earlier termination of the Ca\(^{2+}\) surge, leading to a lower strength of the RAC1/MKK7/JNK/JUN/AP-1 signaling pathway. In such a way, EFN1B1, nAChR, and cell surface testosterone receptor signaling pathways interact among themselves. The sum of their effect determines the outcome of CAT synthesis (Fig. 8).

In the 5’ upstream region of Egr1 gene, there are two groups of serum responsive elements (SRE). The proximal group has 2 SREs at positions +1278/+1295 and +1300/+1313, and the distal group has 3 SREs at positions +974/+992, +1025/+1042, and +1045/+1059 (34). Depending on the cell type, either of the SRE groups has been shown to enhance Egr1 transcription (36,51). ELK1 associates with serum responsive factors (SRF) and the complex binds to SREs of target genes to enhance their transcription (52). ELK1 phosphorylation at S383 and S389 leads to its de-SUMOylation and allows its nuclear translocation and activation (53,54). ERK2 and p38MAPK are the upstream kinases responsible for such phosphorylation (55). We found no difference in the activation of ELK1, ERK2 and p38MAPK, based on their phosphorylation, suggesting that the pathway ERK2 > p38MAPK > ELK1 > SRE in Egr1 transcriptional regulation is not involved in EPHB6-mediated phenotype in AGCCs with regard to catecholamine biosynthesis (Fig. 8).

In summary, with the results of this study, we could construct a hypothetic pathway of EPHB6 > EFN1B1 > RAC1 > MKK7 > JNK > JUN/AP1 > EGR1 > TH > CAT, delineating the regulation CAT biosynthesis by EPHB6, as shown in Figure 8. This pathway merges with the signaling pathway of AChR > Ca\(^{2+}\) > RAC1 > MKK7 > JNK > JUN/AP1 > EGR1 > TH > CAT starting from RAC1, and is necessary for the optimal function of the latter. Deletion of EPHB6 (hence diminished reverse signaling via EFN1B1) compromises this signaling pathway, resulting decreased CAT biosynthesis. The default non-genomic effect of testosterone is to augment K\(^+\) influx, and the absence of EFN1B1 reverse signaling negatively regulates such testosterone effect. In the absence of EPHB6 (hence reduced EFN1B1 reverse signaling), the suppressive effect of testosterone on K\(^+\) efflux is abolished, leading to faster VGCC closure and resulting a negative impact on the Ca\(^{2+}\) > RAC1 > MKK7 > JNK > JUN/AP1 > EGR1 > TH > CAT pathway.

In addition to enhancing our knowledge in the area of chromaffin cell biology, the elucidation of this signaling pathway from EPHB6 to CAT and the interactions among EPHB6, AChR and testosterone in the present study could provide us with potential drug targets in regulating catecholamine biosynthesis, which is implicated in normal and pathological conditions such as blood pressure regulation and Parkinson’s disease. In future studies, gaps in the signaling pathways between EFN1B1 and RAC1, between RAC1 and MKK7, and between EFN1B1 and cell surface androgen receptors need to be further investigated.

**EXPERIMENTAL PROCEDURES**

**Ephb6 gene KO mice**

Ephb6 KO mice were generated in our laboratory, as described previously (7). They were backcrossed to the C57BL/6 genetic background for more than 15 generations. Age- and gender-matched wild type (WT) littermates served as controls. Experiments using castrated mice were
conducted at least 3 weeks post-operation.

**Epinephrine and norepinephrine assays**
The adrenal glands were resected from *Ephb6* KO and WT mice, or castrated KO mice, and were homogenized in 300 µl 0.01 N HCL in the presence of 0.15 mM EDTA. Epinephrine levels in the cleared supernatants were determined by Epinephrine Research ELISA Kits (Rocky Mountain Diagnostics, Colorado Springs, CO, USA, BAE-5100) according to the manufacturer’s instructions. For cultured primary AGCCs, they were pretreated with a cell membrane-impermeable BSA-conjugated testosterone [1.1 µg/ml, testosterone-3-(O-carboxymethyl)-oxime-BSA; testosterone-BSA; Aviva Systems Biology] or BSA for 15 min, and then washed and stimulated with Nicotine (20 µmol/L) for 16 h at 37°C. The cells were washed once with Hank’s buffer and were lysed in 400 µl 0.01 N HCL in the presence of 0.15 mM EDTA. Norepinephrine levels in the cleared supernatants were determined by Norepinephrine Research ELISA Kits (Rocky Mountain Diagnostics). For nonmalignant AGCC line tsAM5NE cells, they were lysed with repeated (3 times) freeze-thaw after being transfected with *siRNAs*. Norepinephrine levels in the cleared supernatants were determined by noradrenaline ELISA kits (LifeSpan BioSciences, Seattle, USA, LS-F10598) according to the manufacturer’s instructions. Samples were assayed in duplicate.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**
mRNA levels of TH, and *Egr1* were measured by RT-qPCR. Total RNA from the adrenal glands medullary cells or tsAM5NE cells was extracted with TRIzol® (Invitrogen, Burlington, Ontario, Canada) and reverse-transcribed with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario, Canada). Supporting Information Table 1 (SI-Table 1) lists the qPCR primers used. qPCR conditions were as follows: 2 min at 50°C, 2 min at 95°C, followed by 40 cycles of 10 s at 94°C, 20 s at 58°C, and 20 s at 72°C. β-actin mRNA levels were considered as internal controls. qPCR signals between 22 and 30 cycles were analyzed. Samples were tested in triplicate, and the data were expressed as signal ratios of target RNA/β-actin mRNA.

**Immunoblotting**
Adrenal gland medullae were isolated from 8- to 10-week old male mice, and cultured in Opti-MEM™ Reduced Serum Media (ThermoFisher scientific, Burlington, Ontario, Canada, Cat. #31985070) at 37°C for 2 hours. In some experiments, nicotine (20 µM) (Sigma-Aldrich, Winston, Ontario, N3876) was used to stimulate the medullae for 2.5 and 5 minutes post the 2-hour culture period. The tissues were then lysed by immunoprecipitation assay buffer (RIPA), which contained PhosSTOP and protease inhibitor mixtures (Roche Applied Science, MeylanCedex, France). For tsAM5NE cells, they were lysed with RIPA buffer 72 hours after being transfected with *Egr1* siRNA or 4 hours after nicotine (40 µM) stimulation in the coated plates. Forty µg of lysate protein per sample was resolved on 10% SDS-PAGE. Proteins were transferred from the gel to PVDF membranes (Invitrogen), which were then incubated in blocking buffer containing 5% (w/v) skim milk or 5% BSA for 1 hour at room temperature. The membranes were incubated overnight at 4°C with rabbit anti-TH Ab (2792; Cell Signaling Technology, Danvers, US), rabbit anti-EGR1 Ab (ab182624; Abcam), rabbit anti-MKK7 (phospho S277+T275) Ab (ab78148; Abcam), rabbit anti-MKK7 Ab (4172; Cell Signaling Technology), rabbit anti-phospho-c-JUN (Ser63) Ab (9261; Cell Signaling Technology), rabbit anti-c-JUN monoclonal Ab (9165; Cell Signaling Technology), rabbit anti-phospho-JNK (Thr183/Tyr185) Ab (9251; Cell Signaling Technology, Danvers, US), rabbit anti-JNK Ab (9252; Cell Signaling Technology), mouse anti-phospho-ERK1/2 (Thr202/Tyr204) Ab (9106; Cell Signaling Technology), rabbit anti-ERK1/2 Ab (9102; Cell Signaling Technology), rabbit anti-phospho-p38MAPK (Thr180/Tyr182) Ab (9211; Cell Signaling Technology), rabbit anti-p38MAPK Ab (9212; Cell Signaling Technology), rabbit anti-phospho-ELK1 (S383) Ab (ab218133; Abcam), rabbit anti-ELK1 Ab (9182; Cell Signaling Technology), or rabbit anti-β-actin Ab (4967; Cell Signaling Technology). Blots were washed and then incubated with horseradish peroxidase-conjugated secondary Abs for 2 hours. All the antibodies were used at the manufacturers’ recommended dilutions. Signals were visualized by SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific).
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**Adrenal gland medulla organ culture**
Adrenal gland medullae were isolated from 8-10-week old Ephb6 KO and WT mice, and cultured in DMEM medium with 15% FCS at 37°C for 0, 2.5 and 5 minutes after nicotine (20 μM) stimulation. The medullae were harvested for the subsequent EMSA or immunoblotting. In some experiments, they were cultured for 4 h in the presence of RAC1 inhibitor (50 μM; 53502, Sigma-Aldrich, Winston, Ontario), MKK7 inhibitor (50 μM; 335140001, Sigma-Aldrich, Winston, Ontario), JNK inhibitor SP600125 (20 μM; S5567, Sigma-Aldrich, Winston, Ontario) or vehicle. The medullae were then harvested for the measurement of Egr1 mRNA levels by RT-qPCR.

**DNA microarray**
Total RNA was extracted using RNeasy Mini Kit (74104, Qiagen, Toronto, Ontario, Canada) from adrenal glands of male, female and castrated male Ephb6 KO mice and their WT counterparts. Three biological replicates using different mice for each group were employed. The RNA was quantified using NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies). Double stranded cDNA was synthesized from 250 ng of total RNA, and in vitro transcription was performed to produce biotin-labeled cRNA using Illumina® TotalPrep RNA Amplification Kit, according to manufacturer’s instructions (Life Technologies). The labeled cRNA was normalized at 1,500 ng and hybridized on MouseWG-6_V2 array according to Illumina’s Whole-Genome Gene Expression Direct Hybridization Assay Guide. The BeadChips were incubated in an Illumina Hybridization oven at 58°C for 16 hours at a standard rocking speed of 5 according to the oven speedometer. Beadchips were washed according to Illumina’s protocol mentioned previously and scanned on an Illumina iScan Reader.

Mean expression signal levels of all the genes of 3 replicates in each group were first obtained. Using the mean expression signal level of each gene of the male WT group for comparison, genes with more than 2-fold changes in their expression in the male KO groups were selected. The fold changes of these selected genes in the female KO group with respect to the female WT group, and the fold changes of these genes in the castrated male KO group with respect to the castrated WT group were calculated and presented.

**GTPase activation assay for RAC1**
Activated RAC1 G-protein within samples (25 μg/sample) was determined by the G-LISA assay (Cytoskeleton, Inc.), performed according to the manufacturer’s instructions. Briefly, adrenal gland medullae were isolated from 8-10-week old Ephb6 KO and WT mice, and cultured in Opti-MEMTM Reduced Serum Media at 37°C for 2 hours. Nicotine (20 μM) was used to stimulate the adrenal medulla for 2.5 minutes, which in pilot studies we determined was the peak activation time. Proteins were extracted from the tissues on ice for 5 minutes in G-LISA cell lysis buffers containing protease inhibitor cocktails (Cytoskeleton, Inc, Denver, Colorado; BK128). The cleared supernatants were snap frozen in liquid nitrogen and stored at -80°C until the assay. Samples were assayed in duplicate. Three independent experiments were conducted and the results were normalized according to the values of WT samples at time 0. The relative RAC1 activity was calculated as follows:

The relative RAC1 activity = RAC1 activity of a given sample/RAC1 activity of WT cells at time 0.

**Chromaffin cell line culture**
AGCC line tsAM5NE cells were cultured in collagen IV-coated 24-well flat bottom plates (Corning, New York, USA, 354430) in DMEM medium with 15% FCS and G5 supplement (ThermoFisher scientific, Burlington, Ontario, Canada, 17503012) in an environment of 5% CO₂ at 33°C. In some experiments, these cells were cultured in wells coated with goat anti-EPHB6 Ab (AF611, R&D systems, Oakville, Ontario, Canada), normal goat IgG (sc-2028, Santa Cruz Biotechnology, Mississauga, Ontario, Canada), recombinant EPHB6-Fc (E9777, Sigma-Aldrich, Oakville, Ontario, Canada), normal human IgG (0150-01, Southern Biotech, Birmingham, USA), rabbit anti-EPNB1 Ab (sc-1011, Santa Cruz Biotechnology), normal rabbit IgG (sc-2027, Santa Cruz Biotechnology), or goat anti-EPNB2...
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Ab (AF496, R&D systems) (2 μg/ml during overnight coating at 4°C) for 24 hours. Nicotine (40 μM) was used to stimulate these cells for 4 hours.

Small interfering RNA (siRNA) transfection
SMARTpool Egr1 siRNA (M-040286-01-0005), which contained 4 pairs of siRNA targeting different regions of Egr1 mRNA, as well as negative control siRNA (D-001206-13-05) were synthesized by Dharmacon (Lafayette, Colorado, USA). The siRNA sequences are listed in SI-Table 2. tsAM5NE cells at a density of 2 x 10^5 cells/well in 24-well plates were transfected with siRNAs (30 nM) with DharmaFECT 1 Transfection Reagent (Dharmacon; T-2001-02) immediately after passage. The transfected cells were cultured for additional 24 to 72 hours before further manipulation.

EMSA and EMSA-immunoblotting
The Ephb6 KO and WT male mice were subcutaneously injected with nicotine in PBS (2 mg/kg body weight) or an equal volume of PBS as a control. The mice were placed in an incubator to prevent hypothermia. After 2 hours, their adrenal medullae were isolated and nuclear proteins were extracted with NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher scientific, Burlington, Ontario, Canada, 78833). EMSA were performed according to the manufacturer’s protocols (Odyssey infrared EMSA kit; LI-COR Biosciences, Lincoln, Nebraska, USA). Duplexed oligonucleotides (5'-GGACTTAGGACTGACCTAGAACAA TCA-3') containing the AP-1-binding sequence (positions +383 to +393) in the 5' untranslated region of mouse Egr1 gene were labeled at 5'-end with IRDye 800 infrared dye (Integrated DNA Technologies, Skokie, Illinois, USA), and used as probes. The extracted nuclear protein (10 μg) was incubated with 5 nM of the IRDye800-labeled probes for 30 minutes. For background control, 200-fold molar excess of unlabeled probes was added to a sample containing WT nuclear extracts to determine the background association of the probes with the nuclear proteins. DNA-protein mixtures were separated by 5% non-denaturing polyacrylamide gel electrophoresis in 0.5 x TBE buffer. Gels were imaged by a LI-COR Odyssey imaging system. The specific AP-1 binding activity was calculated as follows.

The AP-1 binding activity = AP-1 signal of testing samples – signal of the background control

For each experiment, the AP-1 binding activity of WT cells without nicotine stimulation (i.e., treated with PBS) was used to normalize the values of all other samples to calculate their respective normalized relative AP-1 binding activity, using the formula below.

The normalized relative AP-1 binding activity = the specific AP-1 binding activity of a given sample / the specific AP-1 binding activity of WT samples treated with PBS.

To identify c-JUN and c-FOS proteins in the shifted bands, proteins in non-denaturing polyacrylamide gels of EMSA were transferred to PVDF membranes. The membranes were blocked with PBS containing 5% (w/v) skim milk for 1 hour at room temperature. They were then reacted overnight at 4°C with rabbit anti-c-JUN mAb (9165; Cell Signaling Technology), or rabbit anti-c-FOS Ab (ab190289, Abcam). The membranes were washed and then incubated with horseradish peroxidase- conjugated secondary Ab for 2 hours. All the Abs were used at the manufacturers’ recommended dilutions. Signals were visualized by SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific).

Primary AGCC culture
Primary mouse AGCCs were isolated as previously described (23). Briefly, adrenal gland medullae were obtained from 8- to 10-week-old mice. Papain (P4762, Sigma-Aldrich, Oakville, Ontario, Canada) was activated with 5 mM L-cysteine. The medullae were digested by the activated papain in Hank’s buffer at 37°C for 25 min. They were washed twice with Hank’s buffer and then triturated by pipetting in 300 μl Hank’s buffer until they became feather-like. Cells were pelleted at 3,700 g for 3 min and re-suspended in DMEM containing 15% (v/v) FCS for culture. BSA-conjugated testosterone (1.1 μg/ml, testosterone-3-((O-carboxymethyl)-oxime-BSA; Aviva Systems Biology, San Diego, USA) or BSA was added to the culture 15 min prior to the addition of nicotine (20 μM), and the cells were
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cultured for 16 h at 37°C before being harvested for their norepinephrine content measurement.

Ethics statement

ACKNOWLEDGEMENTS

This work was supported by the Fonds de recherche du Québec – Santé to J.W. and H.L. and the J.-Louis Lévesque Foundation to J.W. It was also funded in part by grants from the Canadian Institutes of Health Research to J.W. (MOP272014), the Natural Sciences and Engineering Research Council of Canada (RGPIN-2017-04790) and the Juvenile Diabetes Research Foundation (17-2013-440) to J.W.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.
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FIGURE LEGENDS

Figure 1. Epinephrine content and TH expression in the adrenal glands of WT and KO mice

A. Total epinephrine content in the adrenal glands of WT and KO mice
The results from three independent experiments using different mice were pooled, and the results are shown as means + SD with data points. The data were analyzed by 2-way paired Student’s t test, and the significant p-value is indicated.

B. Similar sizes of KO and WT adrenal glands
Adrenal glands were serially sectioned at an interval of 50 µm at 5 µm thickness. Representative micrographs of the largest HE-stained transection of the gland from each group is presented.

The total volume of the adrenal gland medullae was determined by following formula:

Size of the medulla= mean area of all the sections that contains the medulla × length of the medulla

The results from three individual medullae from different mice of each group were pooled, and the results are shown as means + SD with data points. No significant difference between KO and their WT counterparts is found (2-way paired Student’s t test).

C. TH mRNA expression in adrenal gland medullae of KO and WT mice
Total RNA was extracted from adrenal medullae. TH mRNA level was analyzed by RT-qPCR. β-actin levels were used as internal controls. Samples were measured in triplicate, and the data from three independent experiments using different mice were pooled and expressed as graphs of TH signal/β-actin signal ratios (means + SD with data points). The significant p-value (2-way paired Student’s t tests) is indicated.

D. TH protein expression in the adrenal medullae of KO and WT mice
TH protein levels in the adrenal gland medullae were analyzed by immunoblotting. The representative immunoblotting images are illustrated. The signal ratios of TH versus β-actin were quantified by densitometry. Densitometry data from three or more (as indicated) independent experiments using different mice were pooled and are presented as graphs (means + SD with data points). The significant p-value (2-way paired Student’s t tests) is indicated.

Figure 2. Identification of reduced Egr1 expression in the adrenal glands of male KO mice

A. Significantly altered expression of genes in the cRNA microarray analysis of adrenal gland medullae from KO mice and WT
Total RNA was extracted from adrenal glands and subjected to cRNA microarray analysis. Three biological replicates using difference mice for each group were employed and mean signal levels of each gene in each group were calculated. The genes with their mean signal levels differed above 2-folds between male WT and KO male groups were selected, and the ratios of their mean signal levels were presented in the left column of a log_{10}-scaled heat map. The ratios of the mean signal levels of these selected genes for the female WT versus female KO groups are presented in the middle column, while those between the castrated WT males versus castrated KO males are presented in the right column.

B. Reduced expression of Egr1 mRNA in adrenal medullae from male KO mice
Egr1 mRNA levels in the adrenal gland medullae were analyzed by RT-qPCR. β-actin levels were used as internal controls. Samples in RT-qPCR were in triplicate, and Egr1/β-actin signal ratios from three independent experiments using different mice were pooled and expressed as means + SD with data points. The significant p-value (2-way paired Student’s t tests) is indicated.
C. Reduced Egr1 protein expression in the adrenal medullae of male EPHB6 KO mice

EGR1 protein levels of adrenal gland medullae were analyzed by immunoblotting, and representative images are shown. The intensity of the EGR1 and β-actin bands was measured by densitometry. The results of three independent experiments using different mice were pooled and the signal ratios of EGR1 versus β-actin are presented as graphs (means ± SD with data points). The significant p-value (2-way paired Student’s t tests) is indicated.

Figure 3. Egr1 knockdown by siRNA reduces TH expression and epinephrine content of chromaffin cell line tsAM5NE

Egr1 siRNA and negative control siRNA were transfected into adrenal gland chromaffin cell line tsAM5NE cells.

A. Reduced expression of Egr1 in tsAM5NE cells after Egr1 knockdown

The cells were harvested 24 hours after the Egr1 and control siRNA transfection, and the Egr1 mRNA (left panel) and protein (right panel) levels were determined by RT-qPCR and immunoblotting, respectively. Data from five or more (as indicated) independent experiments were pooled and presented as means ± SD with data points of the ratios of Egr1 mRNA signal versus β-actin mRNA signal, and ratios of EGR1 protein signal versus β-actin protein signal. P-values are indicated (2-way paired Student’s t tests).

B. TH expression was reduced in tsAM5NE cells after Egr1 knockdown

tsAM5NE cells were harvested 72 hours after Egr1 siRNA transfection, the TH mRNA (left panel) and protein (right panel) levels were determined by RT-qPCR and immunoblotting, respectively. Data from five or more (as indicated) independent experiments were pooled and presented as means ± SD with data points of the ratios of TH mRNA signal versus β-actin mRNA signal, and ratios of TH protein signal versus β-actin protein signal. P-values are indicated (2-way paired Student’s t tests). The same membranes were first blotted with anti-EGR1 Ab, and then stripped and re-blotted with anti-TH Ab and anti-β-actin Ab. So the same β-actin immunoblotting was used as loading controls for both Figure 3A and 3B.

C. Reduced noradrenaline content in tsAM5NE chromaffin cells after Egr1 knockdown

tsAM5NE cells were harvested and then lysed 72 hours after Egr1 siRNA transfection. Noradrenaline levels in the cleared supernatants were determined by noradrenaline ELISA, in which samples were measured in duplicate. Results from four independent experiments were pooled and presented as means ± SD with data points P-value is indicated (2-way paired Student’s t tests).

Figure 4. AP-1 level and c-JUN activation in KO and WT adrenal gland medullae

A. Decreased AP-1 association with its binding site in the Egr1 gene in AGCCs of male Ephb6 KO mice

The binding of AP-1 of AGCCs with the AP-1-binding site in the Egr1 gene enhancer was analyzed by EMSA. A representative image is shown at left. The shifted AP-1 bands in the assay are indicated by the bracket. Lane 1: no nuclear protein was added. All other lanes: 10-μg nuclear protein from WT or KO adrenal gland medullae was added to the reaction. Last lane: 200-fold molar excess of unlabeled probe as competitor was added to the reaction to determine the background of the AP-1 binding.

The signals of the shifted AP-1 bands were quantified by densitometry. Specific AP-1 binding signal of each sample was determined by the intensity of the shifted AP-1 band minus the background signal of that region in lane 6. The results were normalized to determine normalized relative AP-1 binding activity (specific AP-1 binding signal of a test sample / specific AP-1 binding signal of WT AGCCs stimulated with PBS). Results of five independent experiments using different mice were pooled, and the normalized relative AP-1 binding signals (means ± SD with data points) are presented in a graph at right. The significant p-value is shown (2-way paired Student’s t test).
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B. Decreased c-JUN phosphorylation in adrenal medullae from male EPHB6 KO mice

Adrenal medullae from male KO and WT mice were isolated and cultured for 2 hours. They were then lysed at 0, 2.5 and 5 minutes after nicotine (20 μM) stimulation. Nuclear proteins from the medullae were extracted. Total and phosphorylated c-JUN levels in the nuclear proteins were determined by immunoblotting. Four independent experiments were conducted. Representative immunoblotting images are shown at left. The signals were quantified by densitometry. The signal ratios of phosphorylated versus total c-JUN, and total c-JUN versus β-actin of four independent experiments using different mice were pooled and are presented as graphs (means ± SD with data points) in the right panels. Significant p-values are indicated (2-way paired Student’s t tests).

Figure 5. EPHB6 signaling pathway upstream of c-JUN

A. Decreased MKK7 phosphorylation in adrenal medullae from male EPHB6 KO mice after nicotine stimulation

B. Decreased JNK phosphorylation in adrenal medullae from male EPHB6 KO mice after nicotine stimulation

For A and B, adrenal medullae from male KO and WT mice were prepared as described in Figure 4B. They were then lysed at 0, 2.5 and 5 minutes after nicotine (20 μM) stimulation. Total and phosphorylated MKK7 and JNK in the lysates were analyzed by immunoblotting. Representative immunoblotting images are shown at left. The signals were quantified by densitometry and the signal ratios of phospho-MKK7 versus total MKK7, total MKK7 versus β-actin, phospho-JNK versus total JNK, and total JNK versus β-actin from four independent experiments using difference mice were pooled and are presented as graphs (means ± SD with data points) in the right panels. Significant p-values are indicated (2-way paired Student’s t tests).

C. Male KO adrenal gland medullae presented reduced RAC1 activity

The adrenal gland medullae were stimulated by nicotine (20 μM) at 37°C for 0 and 2.5 minutes. Activated RAC1 in the tissue was extracted and quantified by a RAC1 G-LISA kit. Samples were assayed in duplicate. Three independent experiments using different mice were performed and the results were normalized according to the formula below.

Normalized RAC1 activity = RAC1 activity of a sample / RAC1 activity of WT medulla at 0 minute

Means ± SD with data points of the normalized RAC1 activity of the three independent experiments are shown. The significant p-value is indicated (2-way paired Student’s t test).

D-F. JNK, MKK7 and RAC1 inhibitors repressed Egr1 mRNA expression in WT adrenal gland medullae

The adrenal gland medullae were isolated from WT male mice. They were cultured in the presence of JNK inhibitor (25 μM), MKK7 inhibitor (50 μM), or RAC1 inhibitor (50 μM) for 4 hours at 37°C. Total RNA from the adrenal gland medullae were isolated and Egr1, β-actin, and Cbl mRNA transcripts were amplified by RT-PCR. Three or more (as indicated) independent experiments using different mice were conducted. The pooled data from these experiments were expressed as means ± SD with data points of signal ratios of target RNA/β-actin mRNA. The significant p-values are indicated (2-way paired Student’s t test).

Figure 6. ERK1/2, p38MAPK and ELK1 were not in the EPHB6 signaling pathway leading to CAT synthesis

Nicotine-stimulated ERK1/2 (A), p38MAPK (B) and ELK1 (C) phosphorylation in adrenal medullae from male KO and WT mice were determined as described in Figure 4B. Representative immunoblotting images are shown at left of each panel. The intensity of the bands was measured by densitometry. The results of three or more (as indicated) independent experiments using different mice were pooled and the signal ratios of phospho-ERK1/2 versus total ERK1/2, total ERK1/2 versus β-actin, phospho-p38MAPK versus total p38MAPK, total p38MAPK versus β-actin, phospho-ELK1 versus total ELK1, and total ELK1 versus β-actin of the KO and WT medullae are presented as graphs (means ± SD with data points). No significant
differences between the KO and WT medullae were found (2-way paired Student’s t tests).

The same membranes were sequentially blotted with anti-phospho-ELK1, ELK1, phospho-ERK1/2, ERK1/2 and β-actin Abs, with stripping process occurred between these different immunoblottings. The same β-actin immunoblotting was used as loading controls for both Figure 6A and C.

Figure 7 EPH/EFN signaling direction and the role of testosterone

A. Reverse but not forward signaling between EPHB6 and EFNBs promotes TH protein expression upon nicotine stimulation

tsAM5NE chromaffin cells were cultured in the 24-well plates coated with anti-EPHB6 Ab, normal goat IgG, recombinant EPHB6-Fc or normal human IgG (2 μg/ml for coating) for 24 hours. They were lysed 4 hours after nicotine (40 μM) stimulation at 33°C. TH protein levels of the cells were determined by immunoblotting. β-actin was used as loading control. Five independent experiments were performed and a representative immunoblot is shown in the upper panel. The signals of the immunoblots were determined by densitometry. The results were normalized by the formula below.

Normalized TH protein expression = ratio of TH/β-actin of cells cultured in different coated wells / ratio of TH/β-actin of cells cultured in wells without any coating

Normalized TH protein expression levels of the five experiments were pooled and their means + SD with data points are presented as a graph in the lower panel. Significant p-values are indicated (2-way paired Student’s t tests),

B. EFNB1 but not EFNB2 promotes TH protein expression in tsAM5NE chromaffin cells upon nicotine stimulation.

The experiments were carried out and the results are presented as described in (A), except that the wells were coated with anti-EFNB1 Ab, normal rabbit IgG, goat anti-EFNB2 Ab or normal goat IgG (2 μg/ml for coating) for 24 hours. Normalized TH protein expression levels of five experiments were pooled and their means + SD with data points are presented as a graph in the lower panel. Significant p-values are indicated (1-way paired Student’s t tests).

C. Non-genomic effect of testosterone on norepinephrine synthesis in AGCCs from female EPHB6 KO and WT mice

Mouse AGCCs were isolated from female EPHB6 KO and WT mice and were cultured in DMEM containing 15 % (v/v) FCS (10,000 cells per well). The cells were pretreated with a cell membrane-impermeable BSA-conjugated testosterone (1.1 μg/ml; Testo-BSA) or BSA for 15 min. Nicotine (20 μM) was then added to the culture. The cells were cultured for 16 h at 37°C, and harvested. They were lysed and norepinephrine levels in the cleared lysates were measured by ELISA. Samples were assayed in duplicate. Norepinephrine content per cell were calculated based on the cell number per well and amount of norepinephrine detected in the lysate of all the cells in a well. The means + SD with data points of norepinephrine levels per cells of three independent experiments are pooled and are presented. Significant p-values are indicated (2-way Student’s t test).

Figure 8. A model of the signaling pathway from EPHB6 to catecholamine biosynthesis in AGCC

This diagram illustrates a hypothetic pathway from cell surface EPHB6 to CAT biosynthesis in AGCCs. EPHB6 exerts reverse signaling through EFNB1, which transduces signals into AGCCs and activates RAC1. RAC1 in turn activates MKK7. MKK7 phosphorylates and activates JNK, which further phosphorylates c-JUN. c-JUN phosphorylation leads to increased nuclear AP-1 association with the AP-1 site in the 5’ untranslated region (+383/+393) of the Egr1 gene. This augments Egr1 expression at both the mRNA and protein levels. EGR1 binds to 3 EGR1-binding sites in the 5’ upstream sequence of the TH gene
at -121/-112, -103/-96 and -50/-41, and augments transcription and translation of TH, the rate-limiting enzyme in CAT biosynthesis. This pathway merges with the signaling pathway of AChR > Ca\(^{2+}\) > RAC1 > MKK7 > JNK > JUN/AP1 > EGR1 > TH > CAT starting from RAC1, and is necessary for the optimal function of the latter. Deletion of EPHB6 (hence diminished reverse signaling via EFNB1) compromises this signaling pathway, resulting decreased CAT biosynthesis. The default non-genomic effect of testosterone is to augment K\(^+\) outflow via the BK channel, leading to a faster closure of VGCC, hence lower total Ca\(^{2+}\) influx triggered by ACh. EFNB1 reverse signaling negatively regulates such testosterone effect. In the absence of EPHB6 (hence reduced EFNB1 reverse signaling), the suppressive effect of testosterone on K\(^+\) efflux is abolished, leading to faster VGCC closure and resulting a negative impact on the Ca\(^{2+}\) > RAC1 > MKK7 > JNK > JUN/AP1 > EGR1 > TH > CAT pathway.

On the other hand, although in the 5’ untranslated region of Egr1 gene, there are 5 SREs, they are not involved in the EPHB6 signaling pathway to TH biosynthesis, because the activation of ELK1, which forms a complex with SRF to become a transcription factor binding to SRE, and the activation of ELK1 upstream kinases ERK2 and p38MAPK, are not affected by EPHB6 deletion. Dark lines with arrows: signaling proven in this study. Blocked grey lines with arrows: theoretically possible signaling based on other studies, but not functional in AGCCs according to our study. TF: transcription factor

Solid dark lines with arrows: signaling proven in this study. Dashed grey lines with arrows: theoretically possible signaling based on other studies or hypothetical signaling pathways suggested by this study. Dashed grey lines with blockage signs: theoretically possible signaling based on other studies, but not functional according to our study. TF: transcription factor; nAChR: nicotinic acetylcholine receptor; VGCC: voltage-gated calcium channel; AR: androgen receptor; small solid circle: Na\(^{+}\); small solid circle: Ca\(^{2+}\); large empty triangle: testosterone.
Figure 1

A

Total Epinephrine content (ng/gland)

|        | Male     | Female   | Castrated male |
|--------|----------|----------|----------------|
| WT     |          |          |                |
| EPHB6 KO |          |          |                |

N=3

B

Total Volume of the Adrenal medulla (mm³)

|        | Male     | Female   | Castrated male |
|--------|----------|----------|----------------|
| WT     |          |          |                |
| EPHB6 KO |          |          |                |

N=3
**Figure 1 Continued**

**C**

Tyrosine Hydroxylase/β-actin mRNA Signal Ratio

- **Male**
  - WT
  - EPHB6 KO
  - N=3

- **Female**
  - WT
  - EPHB6 KO
  - N=3

- **Castrated**
  - WT
  - EPHB6 KO
  - N=3

*p*=0.016

**D**

Tyrosine Hydroxylase/β-actin Protein Signal Ratio

- **Male**
  - WT
  - KO
  - TH
  - 50 KDa
  - β-actin
  - 50 KDa
  - N=8

- **Female**
  - WT
  - KO
  - TH
  - 50 KDa
  - β-actin
  - 50 KDa
  - N=3

- **Castrated**
  - WT
  - KO
  - TH
  - 50 KDa
  - β-actin
  - 50 KDa
  - N=3

*p*=0.031
Figure 2

A

Male WT vs KO  Female WT vs KO  Castrated WT vs KO

| Gene          | Male WT | Female WT | Castrated WT |
|---------------|---------|-----------|--------------|
| EGR1          |         |           |              |
| JUNB         |         |           |              |
| FOS          |         |           |              |
| HSPA1A       |         |           |              |
| DUSP1        |         |           |              |
| PLK3         |         |           |              |
| IER3         |         |           |              |
| ZFP36        |         |           |              |
| AXUD1        |         |           |              |
| LOC666403    |         |           |              |
| VGF          |         |           |              |
| ERDR1        |         |           |              |
| NR4A2        |         |           |              |
| GADD45B      |         |           |              |
| FOSB         |         |           |              |
| LOC100046232 |         |           |              |
| C430002D13R1K|         |           |              |
| MYD116       |         |           |              |
| CFD          |         |           |              |
| DNAJB1       |         |           |              |

Log10 fold changes

Male

\[ p = 0.046 \]

B

\[ EGR1/\beta\text{-actin mRNA Signal Ratio } \]

| Condition | WT | EPHB6 KO |
|-----------|----|----------|
| Male      |    |          |
| Female    |    |          |
| Castrated |    |          |

C

\[ EGR1/\beta\text{-actin Protein Signal Ratio } \]

| Condition | WT | EPHB6 KO |
|-----------|----|----------|
| Male      |    |          |
| Female    |    |          |
| Castrated |    |          |

Figure 2
Figure 3

A

Egr1/β-actin mRNA Signal Ratio

|          | Negative control | Egr1 siRNA |
|----------|------------------|------------|
| p        | 0.003            | 0.001      |

N=5

B

Tyrosine Hydroxylase/β-actin mRNA Signal Ratio

|          | Negative control | Egr1 siRNA |
|----------|------------------|------------|
| p        | 0.001            | 0.001      |

N=5

C

Total Noradrenaline Content (ng/1x10⁶ cells)

|          | Negative control | Egr1 siRNA |
|----------|------------------|------------|
| p        | 0.016            |            |

N=4

Note: The figures show the results of experiments with negative control and Egr1 siRNA treatments, comparing EGR1/β-actin and Tyrosine Hydroxylase/β-actin protein signal ratios along with total noradrenaline content.
Figure 4

A

| Nicotine | No extract | WT | KO | WT | KO | WT | KO |
|----------|------------|----|----|----|----|----|----|
| PBS      | WT         |    |    |    |    |    |    |
| Nicotine | WT         |    |    |    |    | KO |    |

Normalized Relative AP-1 Binding Activity

B

| Nicotine |
|----------|
| Minutes  |
| WT       |
| KO       |
| WT       |
| KO       |

Phospho-c-JUN/c-JUN Ratio

β-actin Ratio

Figure 4
Figure 5

(A) Phospho-JNK/β-actin Ratio

(B) Phospho-MKK7/MKK7 Ratio
Figure 5 Continued

- **C**: Normalized RAC1 Activity over time with nicotine treatment.
  - Nicotine treatment significantly increases RAC1 activity.
  - Comparison of WT and EPHB6 KO groups:
    - WT: p = 0.047
    - EPHB6 KO: p = 0.028
  - N = 3

- **D**: mRNA expression of Egr1 and Cbl.
  - JNK inhibitor treatment affects Egr1 and Cbl expression.
  - N = 3

- **E**: MKK7 inhibitor treatment affects Egr1 and Cbl expression.
  - N = 4

- **F**: RAC1 inhibitor treatment affects Egr1 and Cbl expression.
  - N = 3

Summary:
- Nicotine treatment significantly increases RAC1 activity.
- JNK inhibitor treatment affects Egr1 and Cbl expression.
- MKK7 inhibitor treatment affects Egr1 and Cbl expression.
- RAC1 inhibitor treatment affects Egr1 and Cbl expression.
**A**

| Minutes | WT  | KO  |
|---------|-----|-----|
| 0       | 0   | 0   |
| 2.5     | 2.5 | 2.5 |
| 5       | 5   | 5   |

Phospho-ELK1

ELK1

β-actin

**B**

| Minutes | WT  | KO  |
|---------|-----|-----|
| 0       | 0   | 0   |
| 2.5     | 2.5 | 2.5 |
| 5       | 5   | 5   |

Phospho-p38MAPK

p38MAPK

β-actin

**C**

| Minutes | WT  | KO  |
|---------|-----|-----|
| 0       | 0   | 0   |
| 2.5     | 2.5 | 2.5 |
| 5       | 5   | 5   |

Phospho-ERK1/2

ERK1/2

β-actin

**Figure 6**
**Figure 7**

A

**Nicotine**

| Coated with          | 24 hrs |
|----------------------|--------|
| EPHB6 FC             |        |
| Rabbit IgG           |        |
| Anti-EPHB6 Ab        |        |
| Goat IgG             |        |

Normalized Tyrosine Hydroxylase /β-actin Ratio

- p = 0.039
- p = 0.070

**β-actin**

B

**Nicotine**

| Coated with          | 24 hrs |
|----------------------|--------|
| Anti-EPNB1 Ab        |        |
| Rabbit IgG           |        |
| Anti-EPNB2 Ab        |        |
| Goat IgG             |        |

Normalized Tyrosine Hydroxylase /β-actin Ratio

- p = 0.042
- p = 0.018

**β-actin**
Figure 7 Continued

C

WT KO WT KO
WT+BSA KO+BSA
WT+Testo-BSA KO+Testo-BSA

Nicotine

Norepinephrine content (pg/cell)

WT  KO  WT  KO  WT+BSA  WT+Testo-BSA  KO+BSA  KO+Testo-BSA

Nicotine

N=3

p=0.001
p=0.020
p=0.013
EPHB6 controls catecholamine biosynthesis by up-regulating tyrosine hydroxylase transcription in adrenal gland chromaffin cells
Wei Shi, Yujia Wang, Junzheng Peng, Shijie Qi, Nicolas Vitale, Norio Kaneda, Tomiyasu Murata, Hongyu Luo and Jiangping Wu

J. Biol. Chem. published online March 1, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.005767

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