Regulation of the noradrenaline neurotransmitter phenotype by the transcription factor AP-2β

Seok Jong Hong1, Thomas Lardaro1, Myung Sook Oh1*, Youngbuhm Huh1, Yunmin Ding2, Un Jung Kang2, Jutta Kirfel3, Reinhard Buettner3, and Kwang-Soo Kim1

From the 1Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, the 2Department of Neurology and Neurobiology, Pharmacology & Physiology, University of Chicago, 5841 S. Maryland Avenue, Chicago, IL 60637, and the 3Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127, Bonn, Germany. *Present address: Department of Medicinal Herbology, 312 College of Pharmacy, Kyung Hee University, 1 Hoegi-dong, Dongdaemun-gu, Seoul 130-701, Republic of Korea

RUNNING TITLE: AP-2β regulates the NA phenotype

Address correspondence to: Kwang-Soo Kim, Molecular Neurobiology Laboratory, MRC215, McLean Hospital, Harvard Medical School, 115 Mill Street, Belmont, MA 02478, Tel. 617 855-2024; Fax. 617-855-3479; E-Mail: kskim@mclean.harvard.edu

AP2 family transcription factors are essential for development and morphogenesis of diverse tissues and organs, but their precise roles in specification of neural crest stem cell (NCSC)-derived cell types have not been determined. Among three members known to be expressed in the NCSC (i.e., AP-2α, β, γ), we found that only AP-2β is predominantly expressed in the sympathetic ganglia (SG) of developing mouse embryos, supporting its role in sympathetic development. Indeed, AP-2β null mice expressed significantly reduced levels of both noradrenaline (NA) and NA-synthesizing dopamine β-hydroxylase (DBH) in the peripheral nervous system (PNS). Strikingly, we also found that NA neuron development was significantly compromised in the locus coeruleus (LC) as well. Pharmacological treatment with an NA intermediate during pregnancy significantly rescues the neonatal lethality of AP-2β−/− mice, indicating that NA deficiency is one of the main causes for lethality found in AP-2β−/− mice. We also showed that forced expression of AP-2β, but not other AP-2 factors, in NCSC favors their differentiation into NA neurons. In summary, we propose that AP-2β plays critical and distinctive roles in the NA phenotype specification in both peripheral and central nervous system during development.

A complex regulatory network of extracellular signals and nuclear transcription factors is involved in the specification of neuronal phenotype during development in the vertebrate nervous system (1-3). Neurotransmitter identity is an important feature among the various phenotypes of a particular neuron, because it determines the nature of the chemical neurotransmission a given neuron will mediate, and influences the specific connectivity with target cells. Among various neurotransmitters, NA is one of classical neurotransmitters and controls many essential functions of the nervous system, including memory, attention, emotion, and autonomic function. NA is mainly produced from the LC in the central nervous system (CNS) and the SG in the peripheral nervous system (PNS). NCSCs, which arise from the interface between the neural plate and the surface epidermis, represent a useful paradigm to understand the molecular networks involved in cell fate specification because they give rise to diverse cell types, including the neurons and glia of the PNS, bones, and cartilages (1,4-6).
Using NCSC as the primary experimental system, molecular cascades underlying sympathetic neuronal development and the specification of its NA phenotype have been extensively studied, leading to identification of critical signals and transcription factors such as Mash1/Cash1, Phox2a/2b, dHand, and GATA2/3 (reviewed in (7-9)). Interestingly, most of these transcription factors appear to be unnecessary for the development of NA neurons in the CNS with the exception of Phox2a/2b which are essential for NA neuron development in the LC. However, only Phox2b, not Phox2a, is indispensable for SA development.

The AP2 family proteins are basic helix-span-helix transcription factors that recognize the palindromic 5'-GCCNNNGGC-3' motif or its related GC-rich sequences (reviewed in (10,11)). Since the discovery of AP-2α expressed in the neural crest and neuroectoderm (12,13)), four additional members, AP-2β, AP-2γ, AP-2δ, and AP-2ε, have been added to the AP2 family (14-19). AP2 proteins share unique structures consisting of an N-terminal transactivation domain and a C-terminal DNA binding and dimerization domain. Though AP2 proteins are coexpressed in some developing organs, they show different spatiotemporal expression during development and gene inactivation studies indicated that they may have quite distinct roles in development (11). Based on initial studies of AP-2α and its expression in the neural crest (12, 13), it was generally assumed that AP-2α regulates differentiation of neural crest-derived cells such as sympathoadrenergic (SA) cells. In line with this notion, mutant zebrafish deficient in AP-2α (tfap2a) showed defect in NA neuron development in both the LC and sympathetic neurons (20). However, since AP-2α inactivation in mice causes an early defect leading to neural tube closure, its precise role for NA neuron development was not clearly analyzed in mammals (21, 22).

Because AP-2α, β, and γ are expressed in migrating NCSC (11), we speculated that these three AP2 family members may play important and distinct roles in determination of neural crest-derived cell lineages. To address this, we performed a series of experiments to investigate functional roles of AP-2α, β, and γ in development of neural crest-derived cell types, in particular NA neuron development. Strikingly, our gene expression pattern, loss-of-function, and gain-of-function analyses demonstrate that AP-2β is important for NA neuron development in the PNS of vertebrates. Furthermore, development of NA neurons in the CNS was also dependent on AP-2β. In particular, the lethal phenotype of some AP-2β knockout mice could be pharmacologically rescued, strongly suggesting that the NA deficiency is a proximal cause for the lethality. Taken together, we propose that AP-2β plays important role(s) in the development of NA neurons of both the CNS and PNS.

EXPERIMENTAL PROCEDURES

Animals and DOPS Treatment - Overnight mated female AP-2β mice (C57B6 background) were tested the next morning for the presence of a vaginal plug, which was recorded as gestation day 0.5 (e0.5). Embryos were genotyped by PCR with reverse primer (5’-TTCTCTGAACCTGGGCCACAGTG-3’) and forward primer (5’-TTCTTGGGAGGAATGTCAGTCAAC-3’) or (5’-TGGATGTGGAATGTGTGCGAGG-3’) to detect the wild type or knockout loci of AP-2β, respectively. For NA rescue experiment (23), pregnant mice were treated with 100 μg/ml of L-phenylephrine and L-isoproterenol in the drinking water containing 2 mg/ml ascorbic acid from e8.5 to e16.5. Mice were then administered with DOPS (L-3,4-dihydroxyphenylserine) at 2 mg/ml in the drinking water containing 2 mg/ml ascorbic acid until neonatal day 7 (P7). Toe biopsies were performed on newborn mice for genotyping.
Immunohistochemistry (IHC) and In Situ Hybridization (ISH) - e10.5 to e13.5 mouse embryos were fixed overnight in 4% paraformaldehyde in PBS at 4°C. Embryos were treated with 30% sucrose overnight at 4°C prior to embedding in OCT compound and stored at -70°C. Embryos were sectioned with 16 μm thickness and IHC or ISH were performed. Transverse and parasagittal sections of embryos were used to analyze SG and LC, respectively. Coronal sections of the brain of new born mice were used to analyze LC. Antibodies were detected using the Vectastain kit (Vector Labs) and the signal was visualized using 3,3'-diaminobenzidine (DAB). The following primary antibodies were used: rabbit anti-TH, 1:2,000 for DAB and 1:200 for immunofluorescence (Pel-Freeze, Rodgers, AR); mouse anti-TH, 1:200 for immunofluorescence (Chemicon, Temecula, CA); rabbit anti-AP-2α, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-AP-2β, 1:1,000 (Santa Cruz Biotechnology); rabbit anti-AP-2γ, 1:1,000 (gift from Dr. Eipper), and rabbit anti-Phox2b, 1:10,000 (gift from Dr. Brunet). The specificity of anti-AP-2β antibody was confirmed (supplemental Fig. S1). For immunofluorescence microscopy Alex Fluor 488 or 594 (Molecular Probe, Carlsbad, CA) conjugated antibodies were used after primary antibody treatment. Antisense RNA probes were made from pGEM-Easy T vector (Promega, Madison, WI) in which PCR products were cloned using primer sets described in Table 1. Hybrids with digoxigenin (DIG)-labeled probes were visualized by treatment with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

**Count of Phox2b positive cells and measurement expression of TH and DBH in SG** – To measure the size of SG, regions between forelimb and hindlimb of e13.5 embryos were sectioned with 20 μm thickness. Every tenth section was collected and stained with Phox2b-specific antibodies. Total Phox2b positive cells in the SG from the collected sections were counted. The trunk regions of e13.5 embryos were sectioned with 16 μm thickness. Then, expression of TH and DBH was detected with their specific antibodies and visualized using DAB as described in the previous section. The intensity of signal was measured using NIH image program (ImageJ). At least four different sections in each embryo were analyzed.

**NA Measurement** – e16.5 embryos or brains from newborn mice were frozen and stored at -70°C. Samples were homogenized in the presence of 0.2 M perchloric acid and 0.1 mM EDTA. Samples were disrupted by ultrasonication and centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were extracted with a syringe filter and used to measure the amount of NA by HPLC (high performance liquid chromatography). Pellets were resuspended in phosphate buffer (10 mM potassium phosphate containing 0.2% Triton X-100) and the amount of protein was determined.

**Neural Crest Stem Cell (NCSC) Cultures** - Full-length AP-2α, AP-2β, AP-2γ cDNA was cloned into pSlax13 (24). The cDNAs were cloned into the ClaI site of RCASBP, an avian replication-competent retroviral vector (24). Viruses were made in DF1 chicken fibroblast cells (ATCC) and titers were determined using the anti-gag protein monoclonal antibody (mAb) AMV-3C2 (Developmental Studies Hybridoma Bank (DSHB), Iowa, IA)(25). Primary culture of the trunk region of quail eggs (*Conturnix japonica*, CBT Farms, Chestertown, MD) was performed as described (25). NCSC from the trunk region were infected with each RCAS virus at 5 MOI (multiplicity of infection). After 7 days in culture, neural crest cells were analyzed by quantitative real-time PCR or IHC.

**Quantitative Real-Time PCR Analysis** - cDNA was made from five μg of total RNA, which was isolated using Trizol Reagent (Sigma, St. Louis, MO), using
superscript II (Invitrogen, Carlsbad, CA) with oligo (dT)\textsubscript{12-18} as primer. Real-time PCR analyses were performed in duplicate using SYBR green I using DNA engine Opticon\textsuperscript{TM} (MJ Research, Waltham, MA) to analyze mRNA expression levels. Primer sets used to detect mRNA are shown in Table 1 & 2.

RESULTS

Transcription factor AP-2\textbeta\textsuperscript{β} is specifically expressed in the SG of early chick and mouse embryos - While AP-2\textalpha, \textbeta, and \textgamma are expressed in NCSC, their specific role in neural crest-derived cell lineages are not known. To investigate the potential roles of AP-2 family transcription factors, we analyzed the expression patterns of these factors in developing embryos. Notably, our IHC analyses showed that only AP-2\textbeta was prominently expressed in the SG of e13.5 mouse embryos (Fig. 1A and B), while there were only few AP-2\textalpha expressing cells in the SG (Fig. 1A). Expression of AP-2\textgamma was not detected in the SG (data not shown). This observation is consistent with previous studies (14,26), and strongly suggest that AP-2\textalpha is minimally expressed in the SG, if any. Merging the immunostaining of tyrosine hydroxylase (TH) and AP-2\textbeta showed that their expression almost completely overlapped (Fig. 1B). We next analyzed expression of AP-2\textbeta and other noradrenergic lineage -specific marker genes in early stages of mouse embryo development. Expression of AP-2\textbeta as well as Phox2b, TH, and DBH was detected in the SG, which are formed near to the dorsal aorta, starting from e10.5 (Fig. 1C). These spatio-temporal expression patterns of AP-2\textbeta in the SG of developing embryos prompted us to hypothesize that AP-2\textbeta, among the AP-2 family members, may regulate sympathetic neuron development.

Development and neurotransmitter phenotype specification of NA neurons are compromised in AP-2\textbeta null mice - To further investigate the role of AP-2\textbeta in SA neuronal development as well as in NA phenotype specification \textit{in vivo}, we tested if NA neurons exhibit any developmental abnormalities in the AP-2\textbeta\textsuperscript{−/−} mouse. While previous knockout studies revealed the critical role of AP-2\textbeta in kidney development (27), its role for the nervous system development is not known. We isolated trunk region and hindbrain of embryos to investigate noradrenergic lineage-specific gene expression in the PNS and CNS, respectively. Quantitative real time PCR analysis demonstrated that the expression of DBH mRNA was reduced by approximately 78% in the PNS of AP-2\textbeta\textsuperscript{−/−} embryos compared to their wild type littermates (Fig. 2A). In addition, expression of other NA markers, e.g., GATA-3, Phox2a, and Phox2b, was significantly reduced in the trunk region of AP-2\textbeta\textsuperscript{−/−} embryos (Fig. 2A & data not shown). Analysis of NA maker genes, TH (Fig. 3B, D) and DBH (Fig. 3F), and pan-neuronal markers of the PNS, peripherin (Fig. 3H) and SCG10 (Fig. 3J), indicated that size of the SG in trunk region is reduced in AP-2\textbeta\textsuperscript{−/−} embryos. Gangliogenesis of the SG occurs in a non-synchronized fashion, depending on the location during development. Also the size of SG is various depending on the sectional level. To precisely determine the size of SG, we collected every tenth section of trunk region and counted the total number of NA neurons in the SG by staining Phox2b, which is a NA neuron specific transcription factor. At earlier embryonic stages (i.e., e10.5, e11.5, and e12.5) Phox2b-positive cell counting in the SG of AP-2\textbeta\textsuperscript{−/−} embryos showed 88%, 80%, and 71% of wild type littermates as controls, respectively (data not shown). Analysis of SG of e13.5 mouse embryo trunk region showed that Phox2b positive neurons of AP-2\textbeta\textsuperscript{−/−} embryos were diminished to 40% of wild type littermates (Fig. 3K & supplemental Table S1). However, mRNA level of TH was only marginally affected in the trunk of the embryos (Fig. 2A) although the size of SG
was significantly reduced in AP-2β/- embryos (Fig. 3). This apparent discrepancy is likely because TH is highly expressed in mouse DRG which seems to be intact in AP-2β/- embryos (supplemental Fig. S2)(28). We analyzed the expression level of TH and DBH genes in the NA neurons of SG by comparing the mean intensity (expression level per area), which was calculated using NIH ImageJ program. Expression of DBH in the SG of AP-2β/- embryos was diminished to 62% of wild type littermates (Fig. 3L). While expression of TH in the SG of AP-2β/- embryos was marginally diminished to 83% of wild type littermates (Fig. 3M).

Surprisingly, we found that DBH mRNA expression was even more dramatically diminished in the hindbrain of AP-2β/- embryos (Fig. 2B and Fig. 4). Indeed, DBH was almost non-detectable in the LC of AP-2β/- mice at both e13.5 (Fig. 4D) and P0 (Fig. 4J). In addition, expression of TH and another NA marker, norepinephrine transporter (NET), was significantly diminished in the LC of AP-2β/- embryos (Fig. 4F, L). In line with this observation, we also observed significant decrease of TH mRNA in the hindbrain of AP-2β/- embryos by PCR analysis (Fig. 2B). Most importantly, the level of NA was dramatically diminished (by >95%) in the CNS of mice at both E16.5 and P0 (Fig. 5). NA was also reduced by approximately 30% in the PNS (Fig. 5).

DOPS (L-3, 4-dihydroxyphenylserine) treatment during pregnancy significantly rescues the neonatal lethality of AP-2β/- mice - AP-2β/- mice die during neonatal days 1-2 and this lethality has been attributed to kidney failure (27). In our hands, a small fraction (~10%) of newborn AP-2β/- mice survive beyond P2, but none of them survived past P20 (Fig. 6A; data not shown). We asked whether NA deficiency directly causes the neonatal lethality of AP-2β/- mice. To address this question, we treated pregnant AP-2β/- mice with DOPS, which can be converted to NA by L-aromatic amino acid decarboxylase and allows the animals to bypass the DBH deficiency. Newborn mice were genotyped after birth and their survival was scored each day until P7. As previously reported, the bodyweight of AP-2β/- mice was about 60% of the wild type or heterozygous control littermates at P7 (10). DOPS treatment did not change the body weight of AP-2β/- mice (Fig. 6C & data not shown), suggesting that the body weight defect is not caused by NA deficiency. Strikingly, 100% of AP-2β/- pups survived with DOPS treatment at P0, while 71% survived without DOPS treatment (Fig. 6A). By P4, only 10.4% of untreated AP-2β/- pups survived, while 52% survived with DOPS treatment. Taken together, these results strongly suggest that AP-2β critically regulates the development and neurotransmitter phenotype of NA neurons in both the PNS and CNS of developing mice and that NA deficiency is one of the main causes for neonatal lethality observed in AP-2β/- mice.

Exogenous expression of AP-2β in NCSC favors their differentiation to SA Cells - To further investigate the functional role of AP-2β, we next attempted the gain-of-function approach. Specifically, we tested whether forced expression of AP-2β would distinctively increase the formation of SA cells in quail primary NCSC using avian specific RCAS viruses (25). In parallel, we also tested RCAS viruses which express AP-2α or AP-2γ because they are also known to be expressed in the neural crest. We speculated that NCSC will likely provide the appropriate cellular context because they were previously shown to be able to differentiate into various neural crest derivatives, such as SA neurons and melanocytes (29). Following transduction of NCSC with RCAS viruses, cells were cultured for seven days, and SA cell differentiation was examined by immunocytochemistry and real-time PCR analyses, while differentiation of melanocytes was assessed by the appearance of pigmentation. Forced expression of AP-2β in NCSC resulted in 4-fold increase in
the number of SA cells (Fig. 7G, I) and, correspondingly significant increases in the levels of TH and DBH mRNAs (Fig. 7J), while little difference was observed in the formation of melanocytes. These results corroborate our finding that AP-2β is required for the development of SA cells in our loss-of-function analyses. To our surprise, forced expression of AP-2α in NCSC, on the other hand, greatly diminished the number of TH-positive neurons, but dramatically increased the number of melanocytes in NCSC culture (Fig. 7B, F). Consistent with these observations, mRNA expressions of all NA markers tested (e.g., TH, DBH, and Phox2b) as well as panneuronal markers (e.g., SCG10) were significantly diminished (Fig. 7J and supplemental Fig. S3) by AP-2α expression in NCSC. These results suggest that AP-2α positively controls cell fate determination of NCSC to melanocytes at the expense of SA cells. Meanwhile, forced expression of AP-2γ changed neither the number of TH-positive cells nor mRNA expressions of NA marker genes (e.g., TH, DBH, and Phox2b) nor panneuronal marker SCG10 (Fig. 7D, H, J, and supplemental Fig. S3), indicating that AP-2γ has little effect on differentiation of NCSC into SA neurons. The number of melanocytes was also only marginally affected by AP-2γ.

DISCUSSION

AP-2β critically regulates the NA neurotransmitter phenotype in both the PNS and the CNS of vertebrates - In the nervous system NA controls many essential functions such as memory, attention, emotion, and autonomic function and its abnormal metabolism is implicated in diverse human diseases. Major NA neurons reside in the LC of the CNS and in the SG of the PNS. In this study, we provide several lines of evidence that AP-2β regulates development and phenotype specification of NA neurons in both the CNS and PNS. First, among members of the AP-2 family, AP-2β is predominantly expressed in the SG of mouse embryos and its expression completely coincides with that of the sympathetic neuronal marker, TH. Second, in AP-2β null mice, development of NA neurons appears to be partially defective in the SG of the PNS as well as in the LC of the CNS. In addition, levels of NA, NA-synthesizing enzyme DBH, and the number of NA neurons are significantly diminished in the SG and in the LC. Third, we found that neonatal lethality observed in AP-2β null mice is due, in part, to deficiency of NA because we were able to alleviate their neonatal lethality by treating pregnant females with DOPS. Finally, our forced expression studies showed that AP-2β favors differentiation of chick NCSC to the SA cell fate, while AP-2α appears to play a role in melanocyte differentiation. Taken together, our gene expression studies, loss-of-function, and gain-of-function analyses strongly indicate that AP-2β plays a critical role, probably in conjunction with additional transcription factors (see below), in the development and neurotransmitter specification of NA neurons of both the CNS and the PNS.

Distinctive roles of AP2 transcription factors for differentiation of diverse neural crest-derived cell lineages - The AP2 family transcription factors play essential roles for development and morphogenesis of diverse tissues and organs. In mammalian species, e.g., human and rodents, five members of AP2 factors (α–ε) have been reported (11). Among three AP2 members (AP-2α, β, and γ) expressed in the nervous system, we show that AP-2β specifically regulates development of NA neurons. These results are rather surprising because previous in vitro studies from this and other laboratories showed that AP-2α regulates SA-specific gene promoters such as TH, DBH, and PNMT (30-32). Nevertheless, using the gain-of-function analyses, we found that AP-2α, β, and γ exhibit clearly distinctive roles in cell fate determination of NCSC. While AP-2α
appears to play an important role in melanocyte differentiation from NCSC, AP-2β favors differentiation of NCSC to the SA cell fate. In contrast, exogenous expression of AP-2γ did not affect generation of melanocyte or SA cells. Therefore, it seems that these AP2 family transcription factors may have very distinctive mechanisms in vivo despite the fact that they can similarly transactivate the promoter activities in vitro. Indeed, all three factors (AP2-α, β, and γ) exhibited similar transactivation and DNA binding activities in vitro to the TH and DBH promoters (data now shown). However, we can not exclude the possible role of other AP2 proteins, which may be expressed in specific stage during development, in the differentiation of neural crest-derived SA lineages. Strikingly, our gain-of-function results showed that AP-2α dramatically favors melanocyte formation from NCSC at the expense of SA cells, which is consistent with the recent AP-2α conditional knock-out studies showing that AP-2α is critical for melanocyte differentiation (33). These findings are intriguing when considering the finding that human melanoma is associated with a loss of AP-2α (34) and suggests the possibility that AP-2α may have a crucial role in controlling both differentiation and maintenance of melanocytes. Notably, in the mutant zebrafish lacking AP-2α (tfap2a), NA neurons could not develop properly in both the LC and sympathetic neurons (20). Thus, tfap2a of zebrafish may represent a functional counterpart (orthologue) of AP-2β of vertebrates like mouse for NA neuron development.

Transcriptional regulatory cascade of NA neuron development in the CNS and PNS - During the last decade, important extracellular signals and critical transcription factors controlling NA neuronal cell fate determination, including sympathetic neurons from NCSC, have been identified (1,4,8,9). Such transcription factors include Mash1 (Cash1), Phox2a/2b, GATA2/3, and dHand (7-9,35,36). Among these key transcription factors, Phox2b is unique because it is required for NA neuron development in both the CNS and PNS, rendering it a master regulator of the NA phenotype. In contrast, other factors (e.g., Mash1, GATA3/2, and dHand) appear to be important for NA neurons in the PNS while Phox2a is required for the development of central NA neurons in the LC (7-9,35,37,38). Notably, Phox2b is critical not only for the NA phenotype but also for earlier neurogenesis and patterning (7,9). Thus, the role of Phox2b is not limited to NA neurons but also controls the development of wider structures such as all autonomic ganglia (sympathetic, parasympathetic and enteric), cranial sensory ganglia, adrenal and extraadrenal chromaffin cells, strongly supporting that it co-ordinates general as well as subtype-specific aspects of these neurons as an essential proneural gene. Similarly, other classic basic helix-loop-helix (bHLH) proneural gene such as Olig 2 is known to couple general and subtype-specific identity in motor neuron development (39,40). More recently, another bHLH proneural gene Neurogenin 2 (Ngn2) and the homeodomain factor Lmx1a have been shown to be expressed in proliferating dopaminergic precursors and regulate the development of midbrain dopamine neurons (41-43). Our results indicate that AP-2β is required for proper NA neuron development in both the CNS and the PNS. However, it appears that AP-2β functions at later stages of development and controls more specifically the neuronal subtype identities, instead of general neurogenesis and the pan-neuronal aspects. For example, earlier development of the SG was only marginally defective and its abnormality becomes progressively more evident. Therefore, AP-2β likely coordinates with these transcription factors (e.g., Phox2a/2b, Mash1, GATA3/2, and dHand) to regulate the cascade of NA neuron development in the CNS and PNS. Further investigation of AP-2β function and its ontogenetic relations to upstream signaling molecules and other key transcription factors will provide deeper insights on the regulatory cascade.
underlying the development of NA neurons in the CNS and PNS.

Acknowledgements - We would like to thank Jackie Lee at the University of Colorado at Boulder for critical reading of this manuscript, Jean-François Brunet for anti-Phox2b antibody, Betty Eipper for anti-DBH antibody, Steven Thomas at the University of Pennsylvania and David Robertson at the University of Vanderbilt for the DOPS treatment experiment. This work was supported by NIH grants MH48866 and DC006501 and an International Grant from the Brain Research Center funded by the Ministry of Science and Technology, the Republic of Korea (to KSK), by the Korea Research Foundation Grant (KRF-2006-214-E00037, to MSO), and by a grant from the Deutsche Forschungsgemeinschaft and (to RB).

REFERENCES

FIGURE LEGENDS

FIG. 1. AP-2β is expressed in the SG of early embryonic stages during development. A, Trunk region of e13.5 mouse embryos were stained with antibodies against AP-2α and AP-2β. Scale bar, 100 μm (upper), 200 μm (bottom). B, Co-expression of AP-2β and TH in the SG. The cervical SG of e12.5 mouse embryos were analyzed for gene expression. The same section was stained with rabbit anti-AP-2β and mouse anti-TH specific antibodies. Nuclei were stained with Hoechst dye. Merged image of AP-2β and TH indicates that these genes are co-expressed in the SG. Scale bar, 400 μm (left), 100 μm (right). C, Expression NA lineage specific marker genes during development was detected. Trunk regions of mouse embryos, as indicated, were used for analysis. AP-2β, Phox2b, TH, and DBH were detected by antibody staining. Scale bar, 100 μm. Dorsal root ganglia (DRG), sympathetic ganglia (SG), vertebrae (V) and neural tube (NT) are shown (A, B). SG are indicated by arrowheads (A, C).

FIG. 2. mRNA level of NA-synthesizing DBH is reduced in AP-2β−/− mice. A, B Trunk regions (A) and hindbrain (B), containing NA neurons of the PNS and CNS, respectively, were isolated from e13.5 mouse embryos. Total RNAs were isolated, and quantitative real-time PCR was performed. Each gene expression was normalized according to the expression level of GAPDH. The level of gene expression of AP-2β homozygous mutant mice was compared to that of wild types, which was set at 100% (** P<0.01, *** P<0.001; Student’s t-test). The data represent averages from three e13.5 embryos.

FIG. 3. The expression of DBH is reduced in the SG of AP-2β−/− mice. A-J, Transverse sections of the trunk region of e13.5 embryos are analyzed for gene expression. Representative sections of AP-2β wild type (A, C, E, G, I) and homozygous mutant (B, D, F, H, J) are shown. TH (A, B, C, D) and DBH (E, F) were detected with their specific antibodies. Peripherin (G, H) and SCG10 (I, J) expression was detected by ISH. Vertebrae (V) are shown. SG are indicated by arrowheads (A, B). Scale bar, 100 μm. K, The size of sympathetic ganglia is reduced in AP-2β homozygous mutant embryos. The number of Phox2b positive cells in the trunk region of the SG of AP-2β homozygous mutant mice was compared to that of wild types (e13.5), which was set at 100%. Data are from four littermates. (***P<0.001; Student’s t-test). L, M, The expression of DBH is reduced in the NA neurons of AP-2β homozygous mutant embryos. Mean value (intensity divided by surface area) of DBH (L) or TH (M) expression in the SG of AP-2β homozygous mutant mice was compared to that of wild types (e13.5), which was set at 100%. Data are from three littermates (*P<0.05; Student’s t-test).

FIG. 4. The expression of DBH is reduced in the LC of AP-2β−/− mice. The expression of DBH is reduced in the LC of AP-2β−/− mice. Gene expression of hallmark proteins of NA neurons in LC is shown. A-F, Parasagittal sections of e13.5 AP-2β wild type (A, C, E) and homozygous mutant (B, D, F) embryos were analyzed. G-L, Coronal sections of P0 brains of AP-2β wild type (G, I, K) and homozygote (H, J, L) were used for the analysis. TH (A, B, G, H) was detected by antibody staining. DBH (C, D, I, J) and NET (E, F, K, L) were detected by ISH. Fourth ventricles (IV) are indicated. Scale bar, 200 μm.

FIG. 5. NA contents are reduced in the AP-2β−/− mice. The amount of NA from brains of neonate mice (P0) and head and body of E16.5 embryos were measured by HPLC. Normalized NA contents to the total proteins in AP-2β heterozygous or homozygous mutant mice were
compared with those in their wild type littermates (** P<0.01, ***P<0.001; Student’s t-test). NA contents in wild type are set as 100%.

FIG. 6. **DOPS rescues the lethal phenotype of AP-2β homozygous mutant mice.**  
*The number of AP-2β homozygous mutant mice that survived at P7 was increased by DOPS administration.*  
*Number indicates the number of live or dead, shown in parenthesis, AP-2β−/− mice at the indicated day.*  
*Newborn mice were genotyped at P0 and recorded for their survival at P7 with or without DOPS treatment. Pregnant AP-2β heterozygous mice were treated with L-phenylalanine and isoproterenol from e8.5 to e16.5, and then treated with DOPS until analysis in drinking water.*  
*B, DOPS treated P7 littermates of AP-2β heterozygote and homozygous mutant mice were shown.*  
*C, Body weight of the AP-2β heterozygous and homozygous mutant mice are compared with that of wild type, which was set as 100. Data are averages of ten littermates.*

FIG. 7. **Forced expression facilitates the differentiation of NCSC to the SA cell fate.**  
A-D, *Bright field (BF) images of chick NCSC cultures after 7 days culture following the transduction of viral constructs expressing each of the AP2 proteins; control (A), AP-2α (B), AP-2β (C), and AP-2γ (D).*  
E-H, *TH expression was detected by immuno fluorescence microscopy; control (E), AP-2α (F), AP-2β (G), and AP-2γ (H).*  
*Scale bar, 200 μm.*  
I, *Forced expression of AP-2β increases the number of TH-positive SA cells in NCSC culture. Primary NCSC in (A-H) were stained with TH antibody and Hoechst dye. TH positive cells in total cells per field scored are presented as a percentage.*  
*Data are presented as mean ± SEM from three independent experiments. Ten random fields at 100 X magnification were counted from each culture.*  
J, *Expression of mRNA was measured by quantitative real-time PCR following the transduction of RCAS viruses expressing each of the AP2 proteins. mRNA levels of NA marker genes are normalized to that of GAPDH. For comparison, expression of each gene is shown relative to the expression of the empty RCAS virus infected to NCSC culture (set as 1). The results are from three independent experiments (* P<0.05, ** P<0.01; Student’s t-test).*
FIG. 1.
FIG. 2.

A

Trunk

RNA expression (%)

TH  DBH  GATA-3

0  20  40  60  80  100  120  140

B

Hindbrain

RNA expression (%)

TH  DBH  GATA-3

0  20  40  60  80  100  120  140

+/-

-/-
FIG. 3.
FIG. 4.
FIG. 5.
### Table

<table>
<thead>
<tr>
<th>day</th>
<th>- DOPS</th>
<th>+DOPS</th>
</tr>
</thead>
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<tr>
<td></td>
<td>live (dead)</td>
<td>Survival rate (%)</td>
</tr>
<tr>
<td>P0</td>
<td>69 (27)*</td>
<td>71.8</td>
</tr>
<tr>
<td>P1</td>
<td>30 (39)</td>
<td>31.3</td>
</tr>
<tr>
<td>P2</td>
<td>13 (17)</td>
<td>13.5</td>
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<td>P5</td>
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<td>10.4</td>
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<tr>
<td>P6</td>
<td>10 (0)</td>
<td>10.4</td>
</tr>
<tr>
<td>P7</td>
<td>10 (0)</td>
<td>10.4</td>
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</table>

No. of AP2b (-/-) mice counted: 96

### Figure 6

**A**

<table>
<thead>
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<th>day</th>
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<tbody>
<tr>
<td></td>
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<td>10.4</td>
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<tr>
<td>P7</td>
<td>10 (0)</td>
<td>10.4</td>
</tr>
</tbody>
</table>

No. of AP2b (-/-) mice counted: 96

### B

-/-  +/+

### C

- %

Body weight

-/+  +/-  -/-

**FIG. 6.**
FIG. 7.
**Table 1** Primer sequences used to detect mouse mRNA.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<td>GAPDH</td>
<td>TGACATCAAGAAAGTGTTGAAGC</td>
<td>CCCTGGTTCGCTAGCCGTATTC</td>
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<tr>
<td>TH</td>
<td>CCATTGGAGGCTCTGTTGATTCC</td>
<td>TCACCCCTGCTTGTATTGGAAGG</td>
</tr>
<tr>
<td>DBH</td>
<td>GGAGACTGCCTTTGTGTTGACC</td>
<td>TCCTCACTGCTGAAACTGTTTACC</td>
</tr>
<tr>
<td>GATA-3</td>
<td>TGGAGGTGACCTCGGGACCAG</td>
<td>GCAGGAAGGTGAAAGAGATGAG</td>
</tr>
<tr>
<td>Peripherin</td>
<td>TGATGGATGAAATTGAGTTC</td>
<td>TCGATGTCAGGGCCATCTTG</td>
</tr>
<tr>
<td>SCG10</td>
<td>TCCTCAGCCTGTGGCCTAC</td>
<td>GCAGCTAGATTAGCCCTACG</td>
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</table>

**Table 2** Primer sequences used to detect chick mRNA.

<table>
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<th>Gene</th>
<th>Sense</th>
<th>Antisense</th>
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<tr>
<td>GAPDH</td>
<td>TACACACGGACACTTCAAGGCG</td>
<td>ATGATGTTCCTGGGAGCAGCACCTC</td>
</tr>
<tr>
<td>TH</td>
<td>TGGAAGGAGGTTACAGACACTCTG</td>
<td>TTGTCAGCCAGCCATTGGGAC</td>
</tr>
<tr>
<td>Phox2a</td>
<td>CGTCCGGCTACGATTTCAACC</td>
<td>TGATGCGCAGGGGTCCGGA</td>
</tr>
<tr>
<td>Phox2b</td>
<td>ATGGACACCTCGAGCCTGCTTCA</td>
<td>CTGCGTGAGGTCGATCTTGAGGC</td>
</tr>
<tr>
<td>GATA-2</td>
<td>GCAACCCTTACTATGCCAACCTCTG</td>
<td>TTGATGCTGTCTTTGTCCTCCTTG</td>
</tr>
<tr>
<td>DBH</td>
<td>CACCACATAATCATGTATGAGCCA</td>
<td>GTGTTGAGCTGGGAGGGAAGATG</td>
</tr>
<tr>
<td>Cash1</td>
<td>CGGCTAAACAAGAGATGAGCAAG</td>
<td>GGGAAGAGAAAACGCAACAG</td>
</tr>
<tr>
<td>dHand</td>
<td>CCACGAGGAGAACCCTACTTTC</td>
<td>TTTATCGCTGCTGCTAATCTG</td>
</tr>
<tr>
<td>SCG10</td>
<td>CTGCTCTGTTTTTATCTCTGACC</td>
<td>GCTTTCTCTGCTCCATTGTC</td>
</tr>
</tbody>
</table>
Regulation of the noradrenaline neurotransmitter phenotype by the transcription factor AP-2 β

Seok Jong Hong, Thomas Lardaro, Myung Sook Oh, Youngbuhm Huh, Yunmin Ding, Un Jung Kang, Jutta Kiefel, Reinhard Buettner and Kwang-Soo Kim

J. Biol. Chem. published online April 18, 2008

Access the most updated version of this article at doi: 10.1074/jbc.M709106200

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