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Mice Deficient in both Pituitary Adenylyl Cyclase-activating Polypeptide and Vasoactive Intestinal Peptide Survive, but Display Growth Retardation and Sex-dependent Early Death

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Abstract Pituitary adenylyl cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two closely related neuropeptides exhibiting overlapping activities which have actions on almost every organ system of the body. To determine if these peptides exert essential but redundant functions, we interbred VIP- and PACAP-deficient mice to obtain VIP/PACAP double knockout (DKO) mice. DKO mice had normal birth weights and survived to weaning, but exhibited a dramatic postnatal growth rate reduction. Analyses at postnatal day 16 indicated that all organs examined except the brain were reduced in mass by 40–70% compared to mixed background controls, with the thymus and spleen most profoundly affected. Brain size was also significantly reduced, but by only 10%. The reduced growth rate of DKO mice was associated with reduced serum concentrations of insulin-like growth hormone-1 (IGF-1), but unchanged levels of growth hormone. Despite the normal survival of DKO mice up to the weaning stage, many subsequently experienced early sudden death, with only 48% of females and 82% of males surviving past 6 months. The results indicate that a significant percentage of mice deficient in both VIP and PACAP survive to adulthood, but their growth rate is profoundly affected, and that females in particular exhibit high rate of mortality after about 3 months of age.

Keywords Pituitary adenylyl cyclase-activating polypeptide · PACAP · Vasoactive intestinal peptide · VIP · Knockout · Growth · Postnatal · Dwarf · IGF-1 · Growth hormone

Introduction

Pituitary adenylyl cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) are two closely related neuropeptides in the secretin family (reviewed in Vaudry et al. 2000). VIP and PACAP exist primarily as 28 and 38 amino acid polypeptides, respectively, are 68% identical in the first 28 residues, and exhibit similar high affinity to two mammalian receptor subtypes: VPAC1 and VPAC2. In addition, a closely related receptor, PAC1, preferentially binds PACAP with high affinity, except for a single splice variant which also binds VIP (Lutz et al. 2006). All three receptor subtypes are coupled primarily to G_s-type heterotrimeric G-proteins, and therefore induce cAMP production upon ligand binding. However, there is evidence that other intracellular pathways utilizing PLCβ-PKC, MAP kinase, intracellular Ca^{2+}, and other signaling cascades are in some cases also activated by these peptides. PACAP and VIP have been shown to play important roles in a variety of biological processes, such as inflammation, intestinal function, nervous system development and function, circadian rhythms, and metabolic regulation (reviewed in Delgado et al. 2004; Vaudry et al. 2000). Recent studies

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have examined the essential functions of these peptides through the use of genetically engineered mice. For example, mice with mutations in the genes encoding PACAP (Colwell et al. 2004; Gray et al. 2001; Hamelink et al. 2002; Hashimoto et al. 2001) and VIP (Colwell et al. 2003) have been generated and characterized. PACAP-deficient knockout (KO) mice exhibit significant postnatal lethality (up to 80%) between the first and second week of age due to an apparent metabolic/thermoregulatory defect, but subsequently appear healthy (Gray et al. 2001, 2002). VIP KO mice do not show early lethality, but a small but significant percentage die in the first year due to a paralytic ileus-like condition (Lelievre et al. 2007). Numerous studies demonstrate that although most surviving VIP and PACAP KO mice show no obvious signs of sickness unless challenged, they show a variety of behavioral defects (reviewed in Abad et al. 2006), and have a reduced capacity to reproduce. Despite the reduced survival and other defects, it remains possible that the phenotypes observed in VIP and PACAP KO mice underestimate the action of the lost peptide due to compensation by PACAP or VIP, respectively. A recent study addressed this question and showed no evidence for upregulation of peptides or receptors in extracts of the developing brain (Girard et al. 2006), providing evidence that molecular compensation does not occur in the CNS of these KO strains during development under basal conditions. In order to further investigate the issue of compensation, and to determine if concurrent loss of both peptides is compatible with life, we crossed the two mutant strains to obtain double VIP/PACAP knockout (DKO) mice. We describe some of the phenotypic features of these mice here, with particular emphasis on postnatal growth and survival.

Materials and Methods

Generation of Mice and Monitoring of Growth and Survival All studies were performed within the specific pathogen-free Gonda facility at the University of California at Los Angeles (UCLA). All husbandry and experimental procedures were in compliance with the Animal Welfare Act, Institutional Policies and Guidelines, and adhered to all principles stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources (U.S.) 1996). The experimental protocol was approved by the UCLA Animal Resource Committee. To facilitate the generation of WT and DKO mice on a similar background, previously generated VIP KO (Colwell et al. 2003) and PACAP KO (Colwell et al. 2004) mice on a C57BL/6Jx129Sv background were used. These mice were crossbred in two stages to obtain sets of WT and DKO breeders. Litters of WT and DKO pups for growth studies were then generated from WT×WT, and DKO×DKO interbreedings, respectively, and weaned at 3 weeks of age. The weights of individual pups were determined on a daily basis beginning at birth for up to 40 days. In some cases, assessment of growth was continued on a weekly basis for up to 15 weeks. The health of all mice was monitored on a daily basis, and the dates of death recorded.

Serum Insulin-like Growth Factor-1 (IGF-1) and Growth Hormone (GH) Determinations ELISA assay kits from Diagnostic Systems Laboratories, Inc. (Webster, TX, USA) were used for both IGF-1 (DSL-10-2900) and growth hormone (GH) (DSL-10-72100). Assays were performed on serum from postnatal day 16 mice according to the instructions provided with the kits.

Results

Postnatal Growth of VIP/PACAP Double Mutant Mice is Slower than in Wild-type Mice

Because of the low survival and reduced fertility of PACAP and VIP KO mice on a C57BL/6 background, studies were performed here with PACAP, VIP, and PACAP/VIP double KO (DKO) mice on a mixed C57BL/6Jx129Sv genetic

Fig. 1 WT and DKO pups at postnatal day 16. DKO indicated by (*asterisk*). All mice studied were on a mixed C57BL/6Jx128Sv genetic background

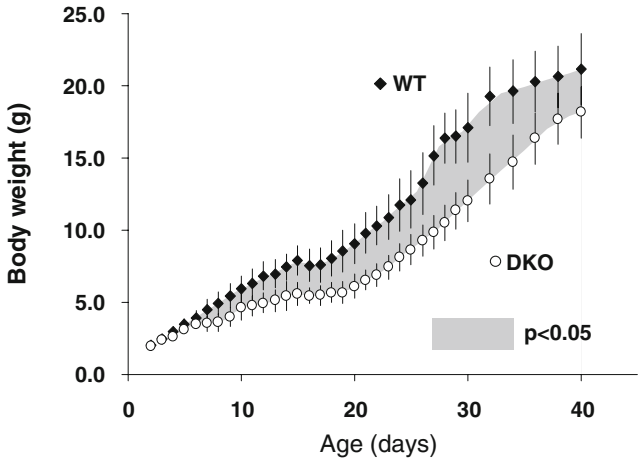

Fig. 2 Growth curves of WT and DKO pups. Weights from WT (*filled diamond*) and DKO (○) pups (n=5 litters each) were recorded daily. *Gray* areas indicate days on which differences were statistically significant (t-test $p<0.05$). All *error bars* represent standard deviation

background, and compared to wild-type (WT) mice of the same mixed background. This strategy was chosen because we have found that both fertility and survival of VIP and PACAP KO mice are greatly increased in this genetic background. Moreover, we found that VIP/PACAP double KO (DKO) mice produced by two-stage cross-breedings of the VIP and PACAP KO mice nearly always survived to weaning (73% and 77%, respectively), and that interbreedings of DKO mice produced viable litters of DKO mice, with a survival rate to weaning of 70% (five litters of each genotype). The most striking initial observation in these studies was that DKO mice exhibited a dwarf phenotype, most obvious around postnatal day (P) 16 (Fig. 1). Thus, we focused our initial studies on comparing the growth rates of DKO and WT mice (n=5 litters each), generated from DKO×DKO and WT×WT pairings, respectively. Although pups from DKO breedings were not significantly smaller than WT pups at birth, they quickly became growth-retarded. By postnatal day 4 (P4) they were significantly smaller than WT pups (p=0.0002) and the difference in body weight continued to increase throughout the first postnatal month (Fig. 2). DKO mice remained significantly smaller than their WT counterparts up to the last day of this initial study (P40). A longer-term study which included five WT and three DKO litters showed that mice subsequently grew in parallel, and that DKO mice were still smaller at 14 weeks (25.1±0.3 DKO vs. 28.4±0.67 WT, mean±SEM, $p<0.01$).

It is unlikely that the observed reduction in growth rate in DKO mice was due to competition of pups, for example, for a limited food supply. Litters used in this study were very similar in size for WT and DKO mice, with a slight tendency towards smaller litters from DKO breedings (data not shown). However, based on growth curve data alone, we could not exclude the possibility that a maternal effect played a role in the observed growth retardation, because DKO pups were generated and raised by DKO mothers. In order to ascertain the contribution of a postnatal maternal

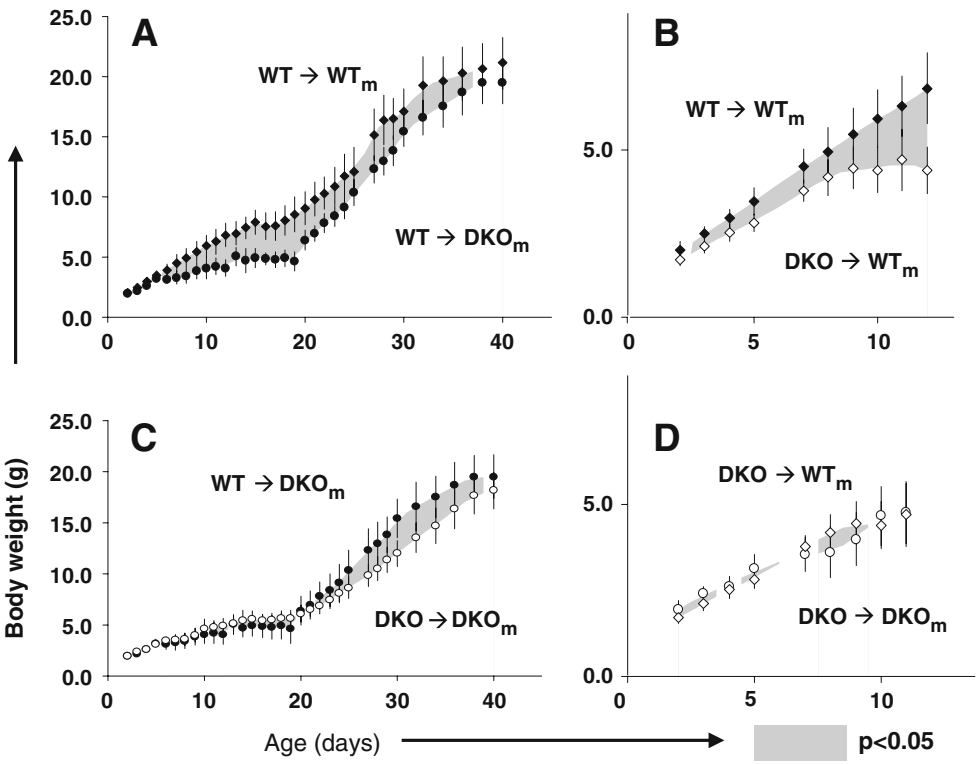

Fig. 3 Growth curves of WT and DKO pups in mother swap experiments. **(a)** WT pups nursed by WT dams compared to WT pups nursed by DKO dams (nine); **(b)** WT pups nursed by WT dams compared to DKO pups nursed by WT dams (*broken bar*); **(c)** WT pups nursed by DKO dams compared to DKO pups nursed by DKO dams; **(d)** DKO pups nursed by WT dams compared to DKO pups nursed by DKO dams. *Gray* areas indicate days on which differences between groups shown in each graph were statistically significant (t-test $p<0.05$). All *error bars* represent standard deviation

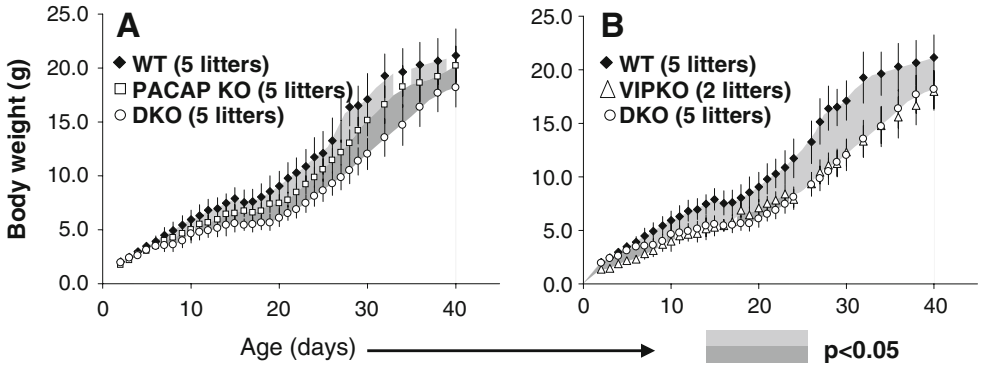

Fig. 4 Growth rate of PACAP and VIP KO mice compared to that of WT and DKO mice. In **(a)** PACAP KO pups (□) are compared to WT (*filled diamond*) and DKO(○) pups; in **(b)** in VIP KO (△) are compared to WT(*filled diamond*) and DKO(○) pups. No surrogate mothers were employed. The number of litters examined is indicated. Growth curves for WT and DKO mice are the same data as in Fig. 3. *Gray* areas indicate days on which differences between groups shown in each graph were statistically significant (*t*-test $p<0.05$).*Two tones of grey* are shown to distinguish single KO from WT or DKO. All *error bars* represent standard deviation. In the case of VIP KO mice, only two VIP KO litters were examined, compared to five from all other genotypes

effect to this phenomenon (for example, providing poor nutrition to pups), we performed mother swap experiments, where pups were moved on P1 to surrogate mothers of the opposite genotype (surrogate mothers had given birth 1 to 3 days prior to that). Two litters of DKO pups transferred to WT mothers died within 2 weeks after birth. Before their death at P12, the body weights of these mice were significantly lower than corresponding weights of WT pups nursed by WT females, the difference becoming progressively more pronounced with time (Fig. 3b). These data suggest that the growth defect in DKO mice could be at least partially ascribed to the genotype of the pups. However, there remained a trend (on some days reaching statistical significance) for DKO pups raised by WT surrogates to be slightly heavier than the DKO pups raised by DKO mothers (Fig. 3d), suggesting a maternal contribution. Moreover, WT pups nursed by DKO mothers showed a similar growth retardation as DKO pups raised by DKO mothers until about P20 (Fig. 3c). Beyond this age, they started growing more rapidly than DKO pups and eventually reached a size significantly higher than DKO pups nursed by DKO mothers (Fig. 3c), and not significantly lower than WT pups nursed by WT mothers (Fig. 3a). The above observations imply that the growth retardation of DKO pups results from both maternal effects (playing a major role in the first 2 weeks after birth) and effects which are nonmaternal, or alternatively, are maternal, but occurring during gestation, but not manifested until after birth.

We then investigated whether or not one of the two genes missing in the DKO mice might contribute more than the other to the observed phenotype. In the course of raising single KO mice (VIP and PACAP) we did not observe any obvious dwarfism in the pups. However, we performed measurements of growth of VIP KO and PACAP KO pups similarly to those for WT and DKO mice. We observed that PACAP KO pups grew significantly slower than their WT counterparts (Fig. 4a), but at later stages of development, this difference became less obvious, becoming not statistically significant by day 40. Moreover, PACAP KO pups grew significantly faster than DKO pups (Fig. 4a). On the other hand, VIP KO mice appeared to exhibit a growth rate defect. These pups grew at a rate similar to DKO pups and significantly slower than WT pups (Fig. 4b). However, the magnitude of the apparent growth defect in VIP KO mice

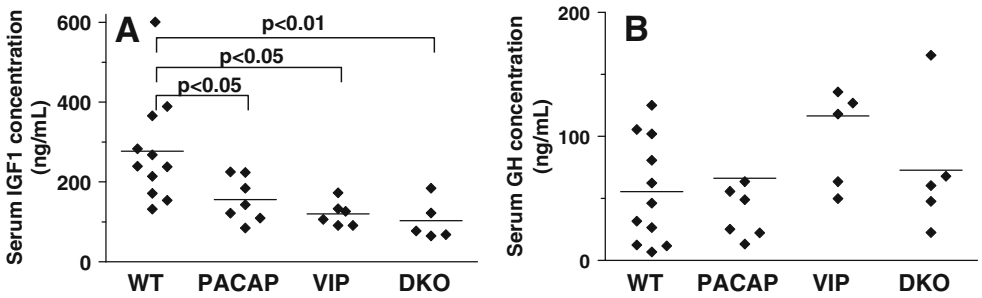

Fig. 5 Serum concentrations of IGF-1 and growth hormone (GH) of P16 pups from indicated genotypes. Measurements IGF-1 (**a**) and GH (**b**) for individual mice are indicated, and *horizontal bars* represent mean values for each group. *t*-test *p* values are indicated, where statistically significant ($p<0.05$). No significant differences were observed for GH

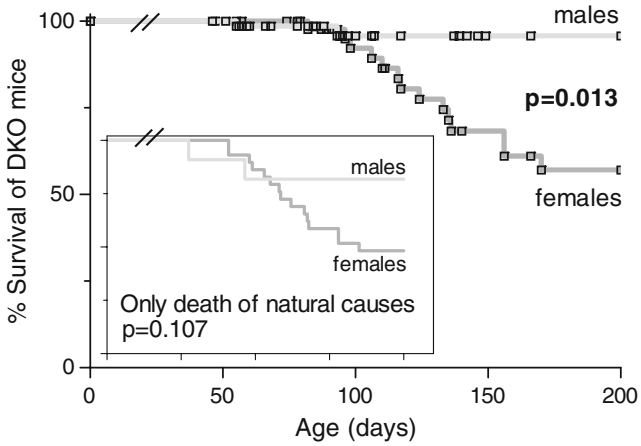

Fig. 6 Kaplan-Meier postweaning survival curves for male and female DKO mice. Main graph: survival curves for all DKO males and females in the colony. *Inset*: survival curves for DKO males and females which died of natural causes. *p* values for Kaplan-Meier test comparing males and females are indicated for each analysis. Mice were excluded that died before weaning (see text for data). No deaths of natural causes were recorded for WT females or males less than 200 days of age during the study period

should be treated with caution, since we only measured the weights of two VIP KO litters, as opposed to five litters for each: WT, PACAP KO, and DKO. Moreover, we have already shown that VIP KO mice on a C57BL/6 background do in fact grow more slowly than control WT mice (Lim et al. 2008), but the difference was not nearly as large as observed in DKO mice.

DKO Mice Show Decreased Levels of Insulin-like Growth Factor, but not Growth Hormone

One of common causes of dwarfism is reduced production and/or secretion of growth factors during infancy. We studied the serum levels of two well-known growth factors: growth hormone, and insulin-like growth factor (IGF-1) at postnatal day 16 in WT, PACAP KO, VIP KO, and DKO mice. While GH levels were not significantly different between genotypes (Fig. 5b), IGF-1 levels were significantly lower in PACAP KO, VIP KO, and DKO mice than in WT mice (Fig. 5a). IGF-1 levels were also lower in DKO compared to VIP KO and PACAP KO mice, but the differences were not statistically significant. Also, even though VIP KO mice had on average lower levels of IGF-1 than PACAP KO mice, the difference was not significant. These data suggest that the loss of VIP certainly contributed to the reduced IGF-1 levels in DKO mice, but the added contribution of PACAP loss is less clear. Most likely, VIP and PACAP have independent and partially additive actions to promote production and/or secretion of IGF-1 into the bloodstream.

DKO Mice Die Prematurely in a Sex-Dependent Manner

In addition to the growth defect in DKO mice, we observed that an unusual number of female DKO mice died suddenly in the period between weaning and 6 months of age. We thus followed a group of 41 DKO mice (30 females and 11 males) who resided in the colony over a full period of 6 months after weaning (21–200 days of age; DOA). Indeed we found that the survival of female DKO mice was reduced in comparison with wild-type mice, with deaths beginning around 3–4 months of age. While natural death before 6 months of age is exceptional in WT mice, only 48% of DKO females and 82% DKO males survived past 6 months postweaning (Fig. 6 inset). There was a clear trend for the females to have higher mortality, but the Kaplan-Meier test did not show significant differences between males and females ($p=0.11$). In order to determine whether this lack of statistical significance was due to insufficient sample size, we performed an additional statistical analysis, which also included survival data on mice which were removed (used for other purposes) before the end of the 6-month study period. This larger analysis, which compared percent survival *while in the colony* of all DKO mice (56 female and 70 male), revealed a statistically significant difference between the survival curves of males and females ($p=0.013$; Fig. 6 main chart). Unfortunately, due to the sudden death of DKO mice, we were unable to

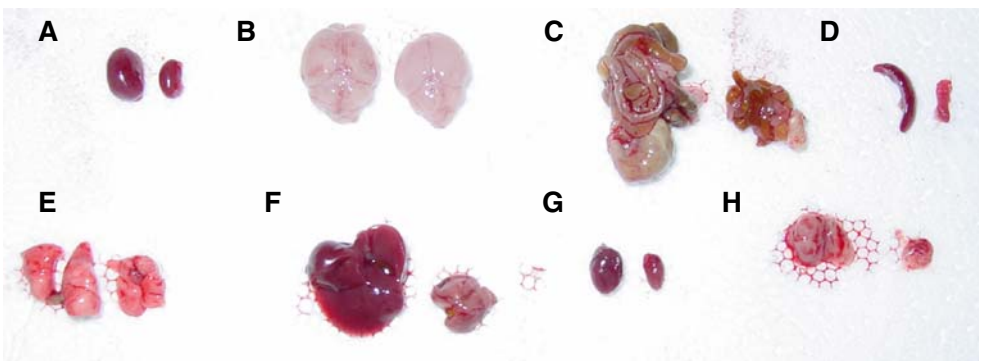

Fig. 7 Organs extracted from P16 WT (*left*) and DKO (*right*) mice: kidney (*A*), brain (*B*), intestine (*C*), spleen (*D*), lung (*E*), liver (*F*), heart (*G*), thymus (*H*)

Table 1 Organ weights and body weight and length of WT and DKO mice at P16

Age=P16	WT			DKO		
	Mean weight (g)	SD	*n*	Mean weight (g)	SD	*n*
Body weight	8.240	0.5547	7	3.679[a]	0.2303	7
Brain	0.438	0.0078	7	0.398[a]	0.0145	7
Lung	0.130	0.0068	6	0.075[a]	0.0119	7
Kidney	0.070	0.0088	12	0.032[a]	0.0035	14
Heart	0.053	0.0033	6	0.021[a]	0.0018	7
Intestine	0.923	0.1181	7	0.317[a]	0.0262	7
Liver	0.418	0.0758	7	0.135[a]	0.0233	6
Thymus	0.073	0.0174	6	0.021[a]	0.0036	6
Spleen	0.048	0.0085	7	0.010[a]	0.0041	6
	Mean length (cm)	SD	*n*	Mean length (cm)	SD	*n*
Body length w/tail	11.571	0.1890	7	9.286[a]	0.2673	7
Body length no tail	6.571	0.1890	7	5.286[a]	0.2673	7

[a] Mean value significantly lower than WT (*t*-test $p<0.001$)

obtain reliable necropsy data to determine the cause of death in these mice.

DKO Mice Show Growth Defects in Multiple Internal Organs

In order to further characterize the phenotype of DKO mice, we dissected organs from P16 WT and DKO mice. All examined organs in DKO mice were significantly smaller ($p<0.0001$), but they did not display gross anatomical abnormalities (Fig. 7). Many organs in DKO showed a proportionally higher decrease in weight than the overall decrease of body weight, thymus and spleen showing most prominent size defect (Table 1; Fig. 8). However, brains in DKO mice seemed normal upon gross examination and showed only a 9% weight decrease in weight compared to WT mice, proportionally much lower than the whole body weight decrease (55%).

Discussion

We describe here the phenotype of mice lacking coding regions for the two closely related polypeptides PACAP and VIP. Most of these double knockout mice survived through weaning (at 21 DOA) and were fertile. Given the multiple important putative biological functions of these peptides, it has been a matter of some debate if the reason for relatively mild phenotypes of single PACAP and VIP knockout mice was that, respectively, VIP and PACAP can substitute for the deleted peptide. However, it appears that even lack of both VIP and PACAP does not cause early postnatal lethality in a mixed C57BL/6Jx129Sv genetic background.

Even though DKO mice generally survive until weaning, they have a severe growth defect, especially pronounced for some internal organs. The causes of such growth retardation may be very diverse in view of pleiotropic character of both VIP and PACAP, and in view of their potential mutually redundant functions in the body. We explored two possible non-exclusive reasons for growth retardation: postnatal maternal effect, i.e., provision of inappropriate maternal care or nutrition before weaning, and impaired growth factor production and/or secretion in pups. We found that DKO pups showed growth retardation despite being reared by a surrogate mother, thus most likely due to innate factors. However, WT pups reared by a surrogate DKO dam also showed growth retardation, which, nonetheless, became less pronounced later in life (around P40). These results suggest that both maternal care and innate factors contribute to the observed phenotype. A further possibility not explored here is that the growth impairment is due in part to the loss of a maternal factor during gestation, as predicted by the studies of Brenneman, Hill, Gressens, and colleagues. Those studies showed that ex vivo treatment of whole mouse E9.5 embryos with VIP greatly accelerated embryonic growth over a 4-h period of study (Gressens et al. 1993),

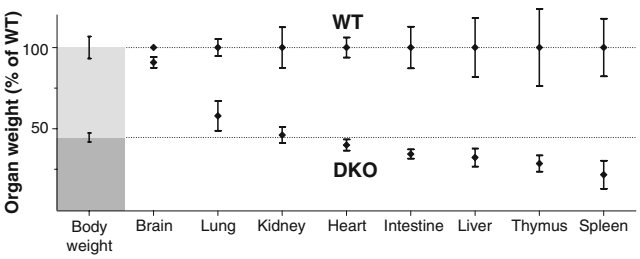

Fig. 8 Organ weights of WT and DKO mice at P16. Values represent mean±SD of % of WT weight for each organ

and that treatment of pregnant mice with a VIP antagonist on gestational day 9–11 resulted in severe microcephaly at birth, albeit with only a 10% reduction in overall birth body size (Gressens et al. 1994) and no significant delay in postnatal growth (Wu et al. 1997). In our studies, the birth weight of DKO pups born from DKO mothers did not differ from that of WT pups born from WT mothers (Fig. 2a), indicating that maternal contribution of neither VIP nor PACAP during embryogenesis is necessary for proper birth weight. However, we did not determine brain sizes in these mice at birth, leaving open the possibility that the birth weight of this organ could have been affected by loss of either maternal or embryo-derived VIP and/or PACAP during gestation.

We measured serum levels of growth hormone and IGF-1 at P16 to determine if either or both of these were affected by loss of either or both neuropeptides. IGFs are thought to be the primary regulators of body growth, acting in a GH-independent manner in the embryo. IGFs continue to act after birth, but appear to become dependent on GH production in mice around 3 weeks of age (Zhou et al. 1997). IGF-receptor-deficient mice are born with a 55% reduction in size, and die shortly thereafter of respiratory failure. GH receptor knockout mice have normal birth weights and grow normally until about 3 weeks, after which a profound growth rate reduction becomes apparent (Zhou et al. 1997). Mice deficient in both VIP and PACAP exhibit a phenotype distinct from IGF and GH receptor KO mice, with a normal birth weight and a growth reduction becoming apparent 4 days after birth (Fig. 2a). Taken with the fact that mean serum GH levels at P16 were unaffected in any of the mutant strains studied here, it can be concluded that the growth suppression observed in DKO mice has little or nothing to do with regulation of GH synthesis or release. Although it is tempting to speculate that VIP and/or PACAP can directly modulate IGF production, it should be taken into consideration that the IGF signaling lies downstream from many processes. For example, undernutrition can profoundly reduce circulating IGF-I and postnatal growth rates in rodents (Desai et al. 1996; Underwood et al. 1994). It is of interest that rat pups nursed by protein-restricted mothers were more than 50% reduced in weight at 21 days compared to those from mothers fed complete diets, but with only a 10–15% decrease in brain size (Desai et al. 1996). Thus, nutritional deficiencies can profoundly affect growth, but do not seem to grossly affect the growth of the brain. With this in mind, it is of interest that metabolic abnormities have been reported in PACAP (Gray et al. 2001), PAC1 (Jamen et al. 2000; Otto et al. 2004), and VPAC2 (Asnicar et al. 2002) KO mice. These were associated with slightly reduced body weights in adult PAC1-deficient mice (Jamen et al. 2000), and a late onset reduction in growth rate of VPAC2 KO mice, beginning at 8–12 weeks of age (Asnicar et al. 2002). Based on all of the data, metabolic defects impinging on IGF signaling in VIP/PACAP double mutant mice provide a plausible mechanism for their dwarf phenotype.

The dramatically reduced survival of female DKO mice beginning at 3–4 months of age was an unexpected finding. PACAP and PAC1 KO mice are known to have reduced survivals, but only due to deaths between the first and second weeks of life (Gray et al. 2001, 2002; Otto et al. 2004). Despite the fact that mice in our studies were monitored on a daily basis, we were unable to collect carcasses in time to perform studies to document the cause of death. Thus, the mechanism of death in these mice and the profound sex dependence remains unknown.

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References

Abad, C., Niewiadomski, P., Loh, D. H. -W., & Waschek, J. A. (2006). Neurotransmitter and immunomodulatory actions of VIP and PACAP: lessons from knockout mice. *International Journal of Peptide Research and Therapeutics*, *12*, 297–310. DOI 10.1007/s10989-006-9032-5.

Asnicar, M. A., Koster, A., Heiman, M. L., Tinsley, F., Smith, D. P., Galbreath, E., et al. (2002). Vasoactive intestinal polypeptide/pituitary adenylate cyclase-activating peptide receptor 2 deficiency in mice results in growth retardation and increased basal metabolic rate. *Endocrinology*, *143*, 3994–4006 Medline DOI 10.1210/en.2002-220354.

Colwell, C. S., Michel, S., Itri, J., Rodriguez, W., Tam, J., Lelievre, V., et al. (2003). Disrupted circadian rhythms in VIP- and PHI-deficient mice. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *285*, R939–949 Medline.

Colwell, C. S., Michel, S., Itri, J., Rodriguez, W., Tam, J., Lelievre, V., et al. (2004). Selective deficits in the circadian light response in mice lacking PACAP. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *287*, R1194–1201 Medline DOI 10.1152/ajpregu.00268.2004.

Delgado, M., Pozo, D., & Ganea, D. (2004). The significance of vasoactive intestinal peptide in immunomodulation. *Pharmacological Reviews*, *56*, 249–290 Medline DOI 10.1124/pr.56.2.7.

Desai, M., Crowther, N. J., Lucas, A., & Hales, C. N. (1996). Organ-selective growth in the offspring of protein-restricted mothers. *British Journal of Nutrition*, *76*, 591–603 Medline DOI 10.1079/BJN19960065.

Girard, B. A., Lelievre, V., Braas, K. M., Razinia, T., Vizzard, M. A., Ioffe, Y., et al. (2006). Noncompensation in peptide/receptor gene expression and distinct behavioral phenotypes in VIP- and PACAP-deficient mice. *Journal of Neurochemistry*, *99*, 499–513 Medline DOI 10.1111/j.1471-4159.2006.04112.x.

Gray, S. L., Cummings, K. J., Jirik, F. R., & Sherwood, N. M. (2001). Targeted disruption of the pituitary adenylate cyclase-activating polypeptide gene results in early postnatal death associated with dysfunction of lipid and carbohydrate metabolism. *Molecular Endocrinology*, *15*, 1739–1747 Medline DOI 10.1210/me.15.10.1739.

Gray, S. L., Yamaguchi, N., Vencova, P., & Sherwood, N. M. (2002). Temperature-sensitive phenotype in mice lacking pituitary adenylate cyclase-activating polypeptide. *Endocrinology*, *143*, 3946–3954 Medline DOI 10.1210/en.2002-220401.

Gressens, P., Hill, J. M., Gozes, I., Fridkin, M., & Brenneman, D. E. (1993). Growth factor function of vasoactive intestinal peptide in whole cultured mouse embryos. *Nature*, *362*, 155–158 Medline DOI 10.1038/362155a0.

Gressens, P., Hill, J. M., Paindaveine, B., Gozes, I., Fridkin, M., & Brenneman, D. E. (1994). Severe microcephaly induced by blockade of vasoactive intestinal peptide function in the primitive neuroepithelium of the mouse. *Journal of Clinical Investigation*, *94*, 2020–2027 Medline DOI 10.1172/JCI117555.

Hamelink, C., Tjurmina, O., Damadzic, R., Young, W. S., Weihe, E., Lee, H. W., et al. (2002). Pituitary adenylate cyclase-activating polypeptide is a sympathoadrenal neurotransmitter involved in catecholamine regulation and glucohomeostasis. *Proceedings of the National Academy of Sciences of the United States of America*, *99*, 461–466 Medline DOI 10.1073/pnas.012608999.

Hashimoto, H., Shintani, N., Tanaka, K., Mori, W., Hirose, M., Matsuda, T., et al. (2001). Altered psychomotor behaviors in mice lacking pituitary adenylate cyclase-activating polypeptide (PACAP). *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 13355–13360 Medline DOI 10.1073/pnas.231094498.

Institute of Laboratory Animal Resources (U.S.) (1996). Guide for the care and use of laboratory animals. Washington, D.C.: National Academy.

Jamen, F., Persson, K., Bertrand, G., Rodriguez-Henche, N., Puech, R., Bockaert, J., et al. (2000). PAC1 receptor-deficient mice display impaired insulinotropic response to glucose and reduced glucose tolerance. *Journal of Clinical Investigation*, *105*, 1307–1315 Medline DOI 10.1172/JCI9387.

Lelievre, V., Favrais, G., Abad, C., Adle-Biassette, H., Lu, Y., Germano, P. M., et al. (2007). Gastrointestinal dysfunction in mice with a targeted mutation in the gene encoding vasoactive intestinal polypeptide: a model for the study of intestinal ileus and Hirschsprung's disease. *Peptides*, *28*, 1688–1699 Medline DOI 10.1016/j.peptides.2007.05.006.

Lim, L. M., Stack, C. M., Cuasay, K., Stone, M. N., McFarlane, H. G., Waschek, J. A., et al. (2008). Regardless of genotype, offspring of VIP deficient female mice exhibit developmental delays and deficits in social behavior. *International Journal of Developmental Neuroscience*.

Lutz, E. M., Ronaldson, E., Shaw, P., Johnson, M. S., Holland, P. J., & Mitchell, R. (2006). Characterization of novel splice variants of the PAC1 receptor in human neuroblastoma cells: consequences for signaling by VIP and PACAP. *Molecular and Cellular Neurosciences*, *31*, 193–209 Medline DOI 10.1016/j.mcn.2005.09.008.

Otto, C., Hein, L., Brede, M., Jahns, R., Engelhardt, S., Grone, H. J., et al. (2004). Pulmonary hypertension and right heart failure in pituitary adenylate cyclase-activating polypeptide type I receptor-deficient mice. *Circulation*, *110*, 3245–3251 Medline DOI 10.1161/01.CIR.0000147235.53360.59.

Underwood, L. E., Thissen, J. P., Lemozy, S., Ketelslegers, J. M., & Clemmons, D. R. (1994). Hormonal and nutritional regulation of IGF-I and its binding proteins. *Hormone Research*, *42*, 145–151 Medline.

Vaudry, D., Gonzalez, B. J., Basille, M., Yon, L., Fournier, A., & Vaudry, H. (2000). Pituitary adenylate cyclase-activating polypeptide and its receptors: from structure to functions. *Pharmacological Reviews*, *52*, 269–324 Medline.

Wu, J. Y., Henins, K. A., Gressens, P., Gozes, I., Fridkin, M., Brenneman, D. E., et al. (1997). Neurobehavioral development of neonatal mice following blockade of VIP during the early embryonic period. *Peptides*, *18*, 1131–1137 Medline DOI 10.1016/S0196-9781(97)00146-0.

Zhou, Y., Xu, B. C., Maheshwari, H. G., He, L., Reed, M., Lozykowski, M., et al. (1997). A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *Proceedings of the National Academy of Sciences of the United States of America*, *94*, 13215–13220 Medline DOI 10.1073/pnas.94.24.13215.