Ephrin-A2 and Ephrin-A5 Are Important for the Functional Development of Cutaneous Innervation in a Mouse Model

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TO THE EDITOR

Eph receptor/Ephrin ligand interactions are important in neuronal mapping and topography in central and peripheral nerves (Palmer and Klein, 2003). All the Eph receptors and Ephrin ligands are expressed in normal human skin (Hafner *et al.*, 2006) and Ephrin-A ligand signaling in hair follicles and keratinocytes has been identified (Yamada *et al.*, 2008). In animal models, sensory neurons express Eph-A receptors, whereas Ephrin-A2 and -A5 have been shown to be important in sensory axonal growth patterning (Muñoz *et al.*, 2005; Walsh and Blumenberg, 2011). Here, we have investigated the role of Ephrin-A2 and -A5 ligands on cutaneous innervation and sensory function. We hypothesized that the loss of either Ephrin-A2 and/or A5 would modify cutaneous innervation and negatively impact sensory function.

All animal studies were approved by the University of Western Australia Animal Ethics Committee. C57BL/6 wild-type, Ephrin-A2$^{-/-}$, Ephrin-A5$^{-/-}$, and Ephrin-A2A5$^{-/-}$ mice were euthanized at day 1 (P1), day 19 (P19), and 3–6 months after birth (adult, $n=5$ per genotype per time point). 1 cm^2 of dorsal skin was harvested and fixed. Nerves were identified by protein gene product (PGP) 9.5 immunohistochemistry and innervation density quantitated (Supplementary Methods; Anderson *et al.* (2010); Morellini *et al.* (2012)). All analysis used one-way analysis of variance and Bonferroni correction for multiple testing.

Both Ephrin-A2 and -A5 ligands are expressed in normal mouse skin epidermis and hair follicles at all time points tested (P1, P19, adult, Supplementary Figure S2 online). Dermal innervation density was not significantly different in any genotype at P1 or adults (Figure 1a and c). Dermal innervation density of Ephrin-A2$^{-/-}$ animals was significantly reduced compared with wild type ($P<0.05$), whereas Ephrin-A2A5$^{-/-}$ mice showed a significant increase in dermal innervation density compared with wild type ($P<0.05$) at P19. Dermal nerve density of Ephrin-A2$^{-/-}$ animals was also significantly decreased compared with Ephrin-A5$^{-/-}$ ($P=0.001$) and Ephrin-A2A5$^{-/-}$ ($P<0.001$) mice at P19 (Figure 1b). Across the age groups wild-type mice did not show a significant difference in dermal nerve density (Figure 2a). Ephrin-A2$^{-/-}$ mice showed a significant increase in nerve density from P19 to adult ($P=0.05$; Figure 2b). Nerve density of Ephrin-A5$^{-/-}$ animals was significantly increased from P1 to P19 ($P<0.05$; Figure 2c). Dermal nerve density of Ephrin-A2A5$^{-/-}$ mice showed a significant increase at P19 compared with P1 ($P<0.01$) and adult mice ($P<0.05$; Figure 2d).

Epidermal innervation of Ephrin-A$^{-/-}$ animals was not significantly different compared with wild-type mice ($P>0.05$) at P1. Epidermal innervation in Ephrin-A5$^{-/-}$ mice was significantly reduced compared with Ephrin-A2$^{-/-}$ animals ($P<0.05$) at P1 (Figure 1d and g–j). Adult Ephrin-A2A5$^{-/-}$ mice also showed a significant reduction compared with wild-type ($P<0.05$), Ephrin-A2$^{-/-}$ ($P<0.05$), and Ephrin- A5$^{-/-}$ mice ($P<0.05$; Figure 1f and o–r). In contrast, epidermal innervation of Ephrin-A2$^{-/-}$ animals was significantly reduced compared with wild-type ($P<0.05$) and Ephin-A5$^{-/-}$ mice ($P<0.001$) at P19 (Figure 1e). Ephrin-A5$^{-/-}$ mice showed a significant increase in epidermal innervation compared with Ephrin-A2A5$^{-/-}$ mice at P19 ($P<0.001$; Figure 1e and k–n).

Epidermal nerve density of adult wild-type mice was significantly reduced compared with P19 wild-type mice ($P<0.05$). Epidermal nerve density of Ephrin-A2$^{-/-}$ animals at P1 was significantly increased compared with P19 ($P<0.01$) and adult mice ($P<0.05$). Epidermal nerve density of Ephrin-A5$^{-/-}$ mice at P19 was significantly increased compared with P1 ($P<0.001$) and adult Ephrin-A5$^{-/-}$ mice ($P<0.001$; Figure 2e–h).

Semmes-Weinstein monofilaments were used to measure sensory function (Supplementary Methods; Chaplan *et al.*, 1994).

Ephrin-A5$^{-/-}$ mice were significantly less sensitive compared with wild-type ($P<0.01$), Ephrin-A2$^{-/-}$ ($P<0.01$), and Ephrin-A2A5$^{-/-}$ ($P=0.05$) mice (Figure 2i). Mean values of 50% paw withdrawal threshold were wild type 0.135 g, Ephrin-A2$^{-/-}$ 0.148 g, Ephrin-A2A5$^{-/-}$ 0.216 g, and Ephrin-A5$^{-/-}$ 0.5 g. There was a trend toward loss of sensory function in A2A5$^{-/-}$ mice. This does not correlate directly with the density of the cutaneous innervation. However, the simultaneous loss of Ephrin-A2 may have an opposing effect to Ephrin-A5 loss and hence a trend but not significant decrease in sensory function in the double knockouts. Aα/β nerve fibers predominantly conduct non-nociceptive sensation (Fang *et al.*, 2005), suggesting Ephrin-A5 is important in the development of Aα/β fibers. However, light touch sensory function of hairy skin is also detected by C-fiber low-threshold mechanoreceptor lanceolate endings (Roudaut *et al.*, 2012). The neuronal staining focused only on interfollicular density in hairy skin, whereas the sen-

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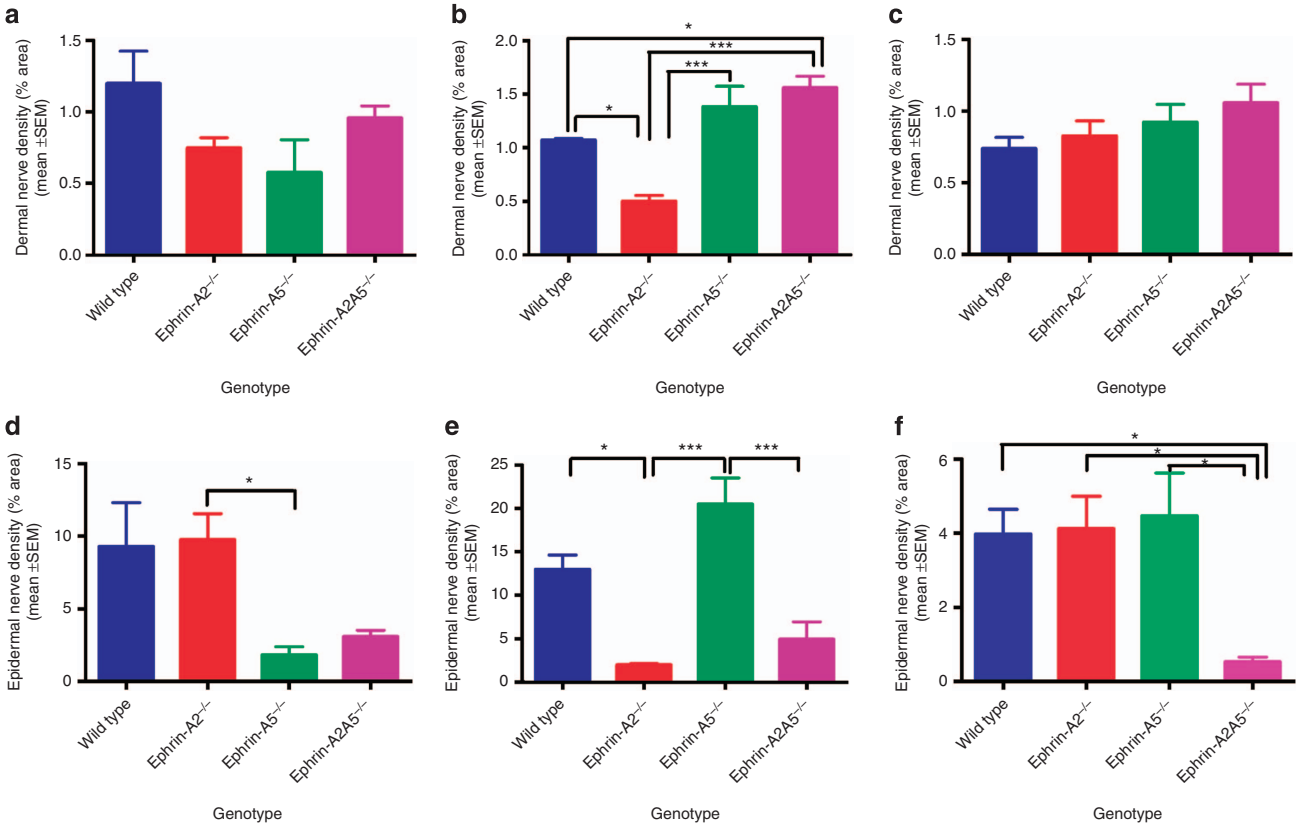
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Dermal nerve density (% area) (mean ±SEM)
Epidermal nerve density (% area) (mean ±SEM)
Wild type
Ephrin-A2−/−
Ephrin-A5−/−
Ephrin-A2A5−/−
Genotype
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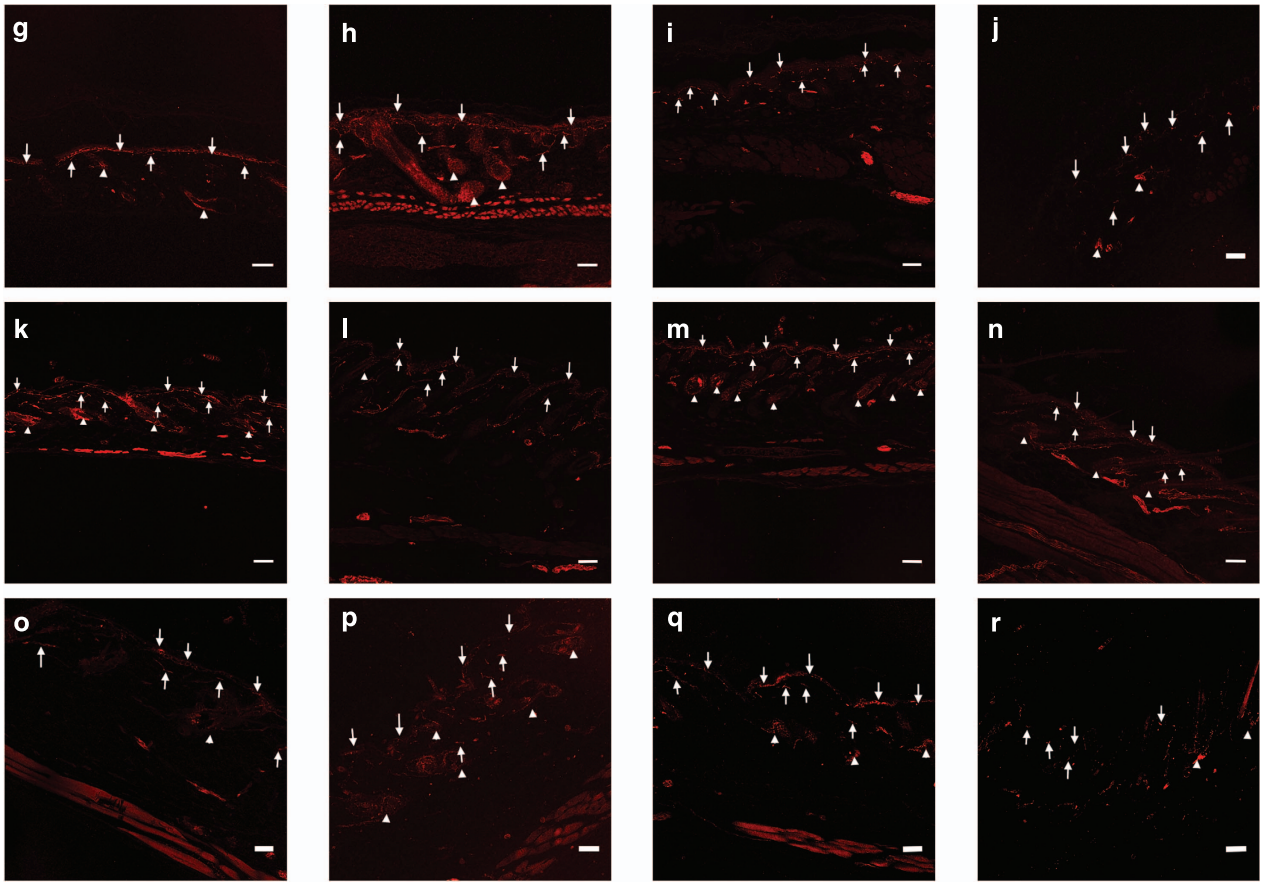
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