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Role of JNK, MEK and adenylyl cyclase signalling in speed and directionality of enteric neural crest-derived cells

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ABSTRACT

Background: Cells derived from the neural crest colonize the developing gut and give rise to the enteric nervous system. The rate at which the ENCC population advances along the bowel will be affected by both the speed and directionality of individual ENCCs. The aim of the study was to use time-lapse imaging and pharmacological activators and inhibitors to examine the role of several intracellular signalling pathways in both the speed and the directionality of individual enteric neural crest-derived cells in intact explants of E12.5 mouse gut. Drugs that activate or inhibit intracellular components proposed to be involved in GDNF-RET and EDN3-ETB signalling in ENCCs were used.

Findings: Pharmacological inhibition of JNK significantly reduced ENCC speed but did not affect ENCC directionality. MEK inhibition did not affect ENCC speed or directionality. Pharmacological activation of adenylyl cyclase or PKA (a downstream cAMP-dependent kinase) resulted in a significant decrease in ENCC speed and an increase in caudal directionality of ENCCs. In addition, adenylyl cyclase activation also resulted in reduced cell-cell contact between ENCCs, however this was not observed following PKA activation, suggesting that the effects of cAMP on adhesion are not mediated by PKA.

Conclusions: JNK is required for normal ENCC migration speed, but not directionality, while cAMP signalling appears to regulate ENCC migration speed, directionality and adhesion. Collectively, our data demonstrate that intracellular signalling pathways can differentially affect the speed and directionality of migrating ENCCs.

1. Introduction

The enteric nervous system arises from cells from multiple axial levels of the neural crest (Le Douarin and Teillet, 1973; Yntema and Hammond, 1954; Uesaka et al., 2015, 2016; Espinosa-Medina et al., 2017). The first wave of cells to colonize the gut arises from neural crest-derived cells that emigrate from the neural tube adjacent to somites 1–7 ("vagal" neural crest) and they colonize the entire bowel. After entering the bowel, enteric neural crest-derived cells (ENCCs) must both populate the gut regions through which they migrate and migrate caudally to ensure there are neurons in all bowel regions. Concomitant population of the migratory route and migration of cells into new regions has been termed "directional dispersion" (Theveneau and Mayor, 2011). It had been assumed that each region of the gut was populated by a sub-population of ENCCs slowing or stopping as other ENCCs progress caudally along the gut into un-colonized regions. However, using time-lapse imaging, we showed that ENCCs in gut regions long populated remain migratory and so each gut region is initially populated by ENCCs migrating non-directionally rather than stopping (Young et al., 2014).

ENCCs colonize the un-populated regions of bowel by expanding the wavefront region of ENCCs (Nishiyama et al., 2012). The rate at which the ENCC population advances along the bowel will be affected by both the speed and directionality of ENCCs within the wavefront. Many mechanisms have been shown to regulate the rate at which the ENCC population colonizes the bowel (McKeown et al., 2013; Obermayr et al., 2013; Avetisyan et al., 2015; Young et al., 2018). Two important signalling pathways influencing the colonisation of the gut by ENCCs are the GDNF-RET (glial derived neurotrophic factor – RET receptor) and

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EDN3-ETB (endothelin 3 - endothelin receptor B) pathways. Knockout mouse models have shown that mice lacking GDNF, RET, or the RET co-receptor GFRα1 have total intestinal aganglionosis (Schuchardt et al., 1994; Sanchez et al., 1996; Pichel et al., 1996; Enomoto et al., 1998), and defects in *EDN3* or *ETB* expression result in colonic aganglionosis (Baynash et al., 1994; Hosoda et al., 1994). Mutations in genes encoding these factors and receptors are also implicated in Hirschsprung Disease, where the failure of ENCCs to colonize the gut results obstruction of the bowel and an accumulation of gut contents in infants, which is fatal if left untreated (reviewed in McKeown et al., 2013; Obermayr et al., 2013; Heanue and Pachnis, 2007; Brosens et al., 2016). Mutations in *Ret* have been identified in 50% of familial Hirschsprung cases (Amiel et al., 2008).

Several intracellular pathways, most of which are thought to be downstream of RET and ETB activation, have also been shown to play a role in ENCC migration (Asai et al., 2006; Barlow et al., 2003; Goto et al., 2013; Natarajan et al., 2002; Stewart et al., 2007). However, it is unclear which intracellular signalling pathways regulate ENCC migration speed and which regulate ENCC migration directionality. Here, we investigated the effect of perturbing the MEK, PKA and JNK intracellular signalling pathways on the migratory speed and direction of individual ENCCs in intact explants of embryonic mouse colon where ENCCs interact with their normal cellular and ECM environment. MEK and PKA have been shown to be downstream of RET and/or ETB activation in the regulation of ENCC migration (Barlow et al., 2003; Goto et al., 2013; Natarajan et al., 2002), while inhibition of JNK has been shown to reduce ENCC migration speed, although it is unclear where JNK sits in the signalling cascades regulating ENCC migration (Asai et al., 2006; Goto et al., 2013). We show that pharmacological inhibition of JNK signalling decreases the speed that individual ENCC migrate without affecting their directionality, while pharmacological activation of PKA or adenylyl cyclase decrease migration speed but increases the caudal directionality of migrating ENCCs.

2. Methods

Time lapse imaging and analysis. Mice carrying E12.5 *Ednrb-hKikGR* mice were killed by cervical dislocation. All ENCCs express KikGR, a photoconvertible protein in *Ednrb-hKikGR* mice (Nishiyama et al., 2012). The colon was removed and set up for time-lapse imaging as described in detail previously (Young et al., 2014; Hao et al., 2019). Only one explant was used in each experiment. The explants were attached to a "V" cut in a piece of filter paper and placed in 2 ml of DMEM/F12 culture medium containing 20 mM glutamine, 10% FBS and penicillin/streptomycin in a 35 mm coverslip-bottomed dish. The dish was then placed in an environmental chamber and imaged on an inverted Leica SP5 laser confocal microscope. Randomly selected ENCCs within 500 μm of the migratory wavefront were photoconverted from green to red. Z-series images at 4.5 μm slice interval were obtained through the ENCC network at 5 min intervals using a X20 oil immersion objective. The preparations were imaged under control conditions for 4–5 h, prior to the addition of drugs to the culture medium. After a 30 min equilibration period, imaging re-commenced and continued for 4–12 h. Cells were tracked manually to determine distance migrated, and measurements of angles and distances were performed on movies of projected z-series using Axiovision (Zeiss, North Ryde, NSW, Australia) software as described previously (Young et al., 2014) (see Fig. 1). For direction of migration, the long axis of the colon in the caudal direction was defined as 0°. For caudally migrating ENCCs, this is a more meaningful measure of directionality than persistence, which measures deviation of path distance from linear distance regardless of direction of migration. To compare ENCC speed between control and drug conditions, *t* tests were performed. To compare the angle of migration of ENCCs, Watson's U^2 test for angles was performed (Zar, 1999). All experiments were approved by the Anatomy & Neuroscience, Pathology, Pharmacology and Physiology Animal Ethics Committee of the University of Melbourne.

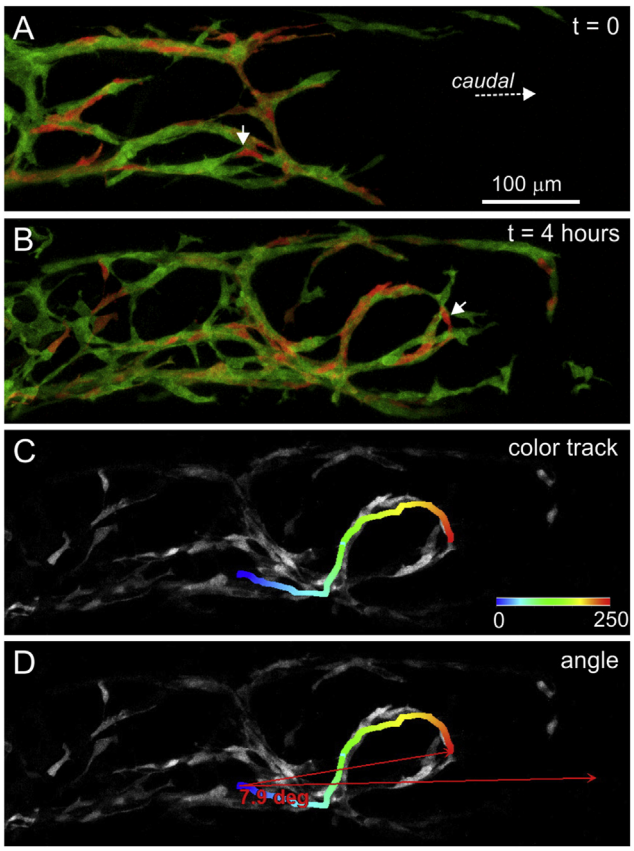

Fig. 1. *Determination of speed and directionality of individual ENCCs in intact explants of colon from an E12.5 Ednrb-hKikGR mouse.* **A.** Stacked confocal micrograph of the ENCC migratory wavefront in an E12.5 colon explant in which a random sub-population of ENCCs had been photoconverted from green to red (an example is *arrowed*). Photoconverted cells that remained in the field of view for a minimum of 1 h were tracked and their speed and directionality determined. **B.** The location of the cell arrowed in A is shown 4 h later (*arrow*). **C.** The tracked path followed by the cell with distance migrated indicated by different colors (see bar in bottom corner). This cell migrated 250 μm in 4 h. **D.** The angle between the start and end locations of the cell was measured relative to the long axis of the explant. This cell migrated caudally at an angle of 7.9°.

Drugs. The following drugs were used to perturb intracellular signalling pathways: JNK inhibitor, SP600125 (30 μM); MEK inhibitor, PD184352 (10 μM); adenylyl cyclase activator, forskolin (100 μM); activator of PKA, SP-8-Br-cAMP (120 μM); inhibitor of cAMP-dependent kinases, RpcAMPs (50 μM, 500 μM). All drug compounds were purchased from Sigma Aldrich. Drugs were prepared as 1000-fold stock solutions dissolved in distilled water (RpcAMPs) or DMSO (other drugs) and then 2 μl of stock drug solution was added to 2 ml of tissue culture medium. Control experiments showed that 1:1000 DMSO or distilled water had no effect on speed or direction of ENCCs. Drug concentrations were based on previous studies (Asai et al., 2006; Goto et al., 2013; Oh et al., 2017; Wu et al., 2016). A minimum of 5 experiments were performed for each drug condition; each experiment was performed on tissue from a different mouse litter on a different day. Due to the time-consuming manual tracking, and the very large sample size needed to obtain statistically robust data due to the variability between individual ENCCs in speed and behaviour, it was not feasible to perform dose-response experiments.

Immunohistochemistry. Immunohistochemistry was performed as described previously (Hao et al., 2017) using the following antisera: human anti-HuC/D (1:5000; Gift of Dr Vanda Lennon) (Fairman et al., 1995), goat anti-Sox 10 (1:300, Santa Cruz, sc-17342), and rabbit anti pCREB (1:100, Cell Signalling Technology). The secondary antisera used were donkey anti-sheep Alexa 594 (1:100, Invitrogen), donkey

anti-human Alexa 647 (1:200, Jackson) and donkey anti-rabbit FITC (1:200, Jackson).

3. Results and discussion

The effects of pharmacological perturbation of particular intracellular signalling pathways on ENCC speed and directionality was examined in intact explants of colon from E12.5 mice using timelapse imaging. At this age, the migratory wavefront of ENCCs is in the proximal colon (Obermayr et al., 2013). The entire colon from E12.5 *Ednrb-hKikGR* mice, in which all ENCCs express the photoconvertible protein, KIKGR (Nishiyama et al., 2012), was dissected. Random ENCCs within 500 μm of the migratory wavefront were photoconverted from green to red, which enabled individual ENCCs to be discerned, and then the explants were imaged at 5 min intervals using a confocal microscope. ENCC speed and directionality of individual ENCCs were measured from the movies as described previously (Young et al., 2014) in explants grown in control conditions and in the presence of drugs affecting specific intracellular signalling pathways. As reported previously, the speed and directionality of individual ENCCs was very variable (Young et al., 2014). The cause of the variability in migratory behaviour is currently unknown, but could reflect the action of multiple mechanisms influencing ENCC migration.

We did not investigate the effects of the drugs on ENCC proliferation because the length of the cell cycle of ENCCs is approximately 20 h at E12.5 (Gonsalvez et al., 2015), and our recording time of 4–12 h is therefore too short to capture sufficient cell divisions. We did not observe cell death in any of the control or experimental movies, apart from in the presence of forskolin (described below).

Effects of JNK inhibition on speed and directionality of ENCCs. The effects of JNK inhibition of ENCC speed and directionality were examined using the JNK inhibitor, SP600125 (30 μM). Confirming an earlier report (Goto et al., 2013), JNK inhibition significantly decreased the speed of migration of individual ENCCs (Fig. 2A). Interesting, however, Goto et al. (2013) were unable to show a significant correlation between JNK activity measured using FRET biosenses and ENCC migration speed. JNK inhibition had no significant effect on the angle of migration of ENCCs (Fig. 2A). We have previously reported that pharmacological inhibition of endothelin receptor B (ET-BR) signalling decreases ENCC speed without significantly affecting caudal directionality (Young et al., 2014); the current data confirm that speed and caudal directionality of ENCCs can be regulated independently. Downstream of RET, multiple interactions between pathways, including JNK, have been proposed (Gerits et al., 2008), however, Goto et al. (2013) did not detect JNK activation in cultured ENCCs exposed to exogenous GDNF or EDN3. Thus, although we have confirmed a role for JNK in promoting ENCC migration, the upstream and downstream signalling events involving JNK that promote ENCC migration are unclear.

Effects of MEK inhibition on speed and directionality of ENCCs. In explants of embryonic gut cultured on a collagen gel matrix, inhibition of the MAPK/ERK pathway decreases the number of ENCCs that migrate away from the explant towards a source of the ENCC chemoattractant, GDNF (Natarajan et al., 2002). We therefore examined the effects of the MEK inhibitor, PD184352 (10 μM), on the speed and directionality of ENCCs in intact explants. Following MEK inhibition, there was no significant change in the speed or directionality of ENCCs (Fig. 2B). Our data are consistent with two earlier studies that also found that MEK inhibition did not reduce the speed of migrating ENCCs in intact gut explants (Asai et al., 2006; Goto et al., 2013). This is the first investigation of the influence of MEK inhibition on ENCC directionality.

The differences between the effects of MEK inhibition on cultured ENCCs migrating towards a source of GDNF on collagen gels compared to ENCCs migrating intact gut explants shows that the mechanisms regulating ENCC migration are context-dependent. We have reported previously that pharmacological inhibition of ROCK-I/II reduces the speed at which the ENCC population migrates in intact gut explants but increases ENCC migration speed in collagen gels containing GDNF (Stewart et al., 2007). Furthermore, pharmacological inhibition of ET-BR in intact explants of embryonic gut (Young et al., 2014; Druckenbrod and Epstein, 2009; Nagy and Goldstein, 2006; Sidebotham et al., 2002) and mutations in genes encoding EDN3 or ET-BR (Baynash et al., 1994; Kapur et al., 1992, 1993) result in delayed ENCC migration, showing that ET-BR signalling promotes ENCC migration within the normal gut environment, but when cultured on collagen gels, the migration of ENCCs towards a source of GDNF is inhibited by EDN3 (Barlow et al., 2003). Our data confirm that the MAPK/ERK pathway is not essential for ENCC migration in their normal environment.

Effects of perturbations to the PKA pathway on speed and directionality of ENCCs. Mice with a mutation at a putative PKA phosphorylation site of Ret show delayed ENCC migration along the gut in vivo (Asai et al., 2006). Furthermore, both pharmacological inhibition of PKA (Asai et al., 2006) and exposure to a cAMP analogue (Goto et al., 2013) have been reported to slow ENCC migration in intact explants of embryonic gut suggesting that an optimal level of cAMP signalling is required for normal ENCC migration speed. The role of cAMP signalling on ENCC directionality had not previously been investigated. We therefore examined the effects of various pharmacological perturbations to the PKA signalling pathway on the speed and directionality of individual ENCCs (Fig. 3).

We first examined the effects of the PKA activator, SP-8-Br-cAMP (120 μM), on ENCC migration. Confirming an earlier report (Goto et al., 2013), SP-8-Br-cAMP significantly decreased the speed of migration of individual ENCCs (Fig. 2C). SP-8-Br-cAMP also influenced the directional migration of ENCCs as there was a significant increase in the proportion of ENCCs migrating caudally (Fig. 2C) in its presence; in control preparations, 58% of ENCCs migrated caudally (had a migration angle of $<90^\circ$ or $>270^\circ$; the long axis of the gut was defined as 0°), whereas in SP-8-Br-cAMP-treated preparations, 81% of ENCCs migrated caudally (had a migration angle of $<90^\circ$ or $>270^\circ$) (Fig. 2). A gradient of GDNF along the developing bowel has been proposed to drive the directional migration of ENCCs (Natarajan et al., 2002), and it has been shown that PKA is required for the chemotactic response of ENCCs grown on collagen gels to a source of GDNF (Asai et al., 2006). Combined with our data, it is possible that GDNF produced by the gut mesenchyme induces activation of PKA in ENCCs, which promotes their caudal directional migration. However, this idea is difficult to reconcile with a study that used mice expressing FRET biosensors, which showed that GDNF inhibits PKA in ENCCs (Goto et al., 2013), so it is also possible that environmental cues other than GDNF regulate PKA levels in ENCC and their directional migration.

We then examined the effects of the adenylyl cyclase activator, forskolin (100 μM). Like the PKA activator, SP-8-Br-cAMP, forskolin decreased ENCC speed but did not completely halt migration. Forskolin also increased the proportion of ENCCs migrating caudally (Fig. 2D); 59% of ENCCs migrated caudally (had a migration angle of $<90^\circ$ or $>270^\circ$) in controls, whereas in forskolin-treated preparations, 66% of ENCCs migrated caudally (Fig. 2D). However, unlike SP-8-Br-cAMP, after about 4 h exposure to forskolin, the morphology of the ENCC network changed and there was significantly less cell-cell contact (Fig. 4). The density of solitary ENCCs, defined as KikGR-expressing ENCCs without detectable contacts with other ENCCs, was quantified following 12 h exposure to forskolin, and there was a >3-fold increase (145 ± 33 solitary cells/mm^2 following 12 h exposure to forskolin compared to 40 ± 8 cells/mm^2 in time controls, $n = 5$, unpaired t-test, $p < 0.05$). The change in morphology was not associated with a dramatic increase in ENCC death; frame-by-frame analyses of movies of a control preparation and a forskolin-treated preparation exposed to forskolin for 12 h revealed 0 dying cells in the control preparation and 2 dying cells in the preparation exposed to forskolin. Previous studies have shown that cell-cell contact between ENCC cells is important for migration (Young et al., 2004, 2014; Anderson et al., 2006). As we showed that PKA activation did not have any detectable effects on ENCC adhesion, our data suggest that cAMP levels are likely to regulate ENCC cell-cell adhesion independently of PKA. Epac, another target of cAMP, has been shown to

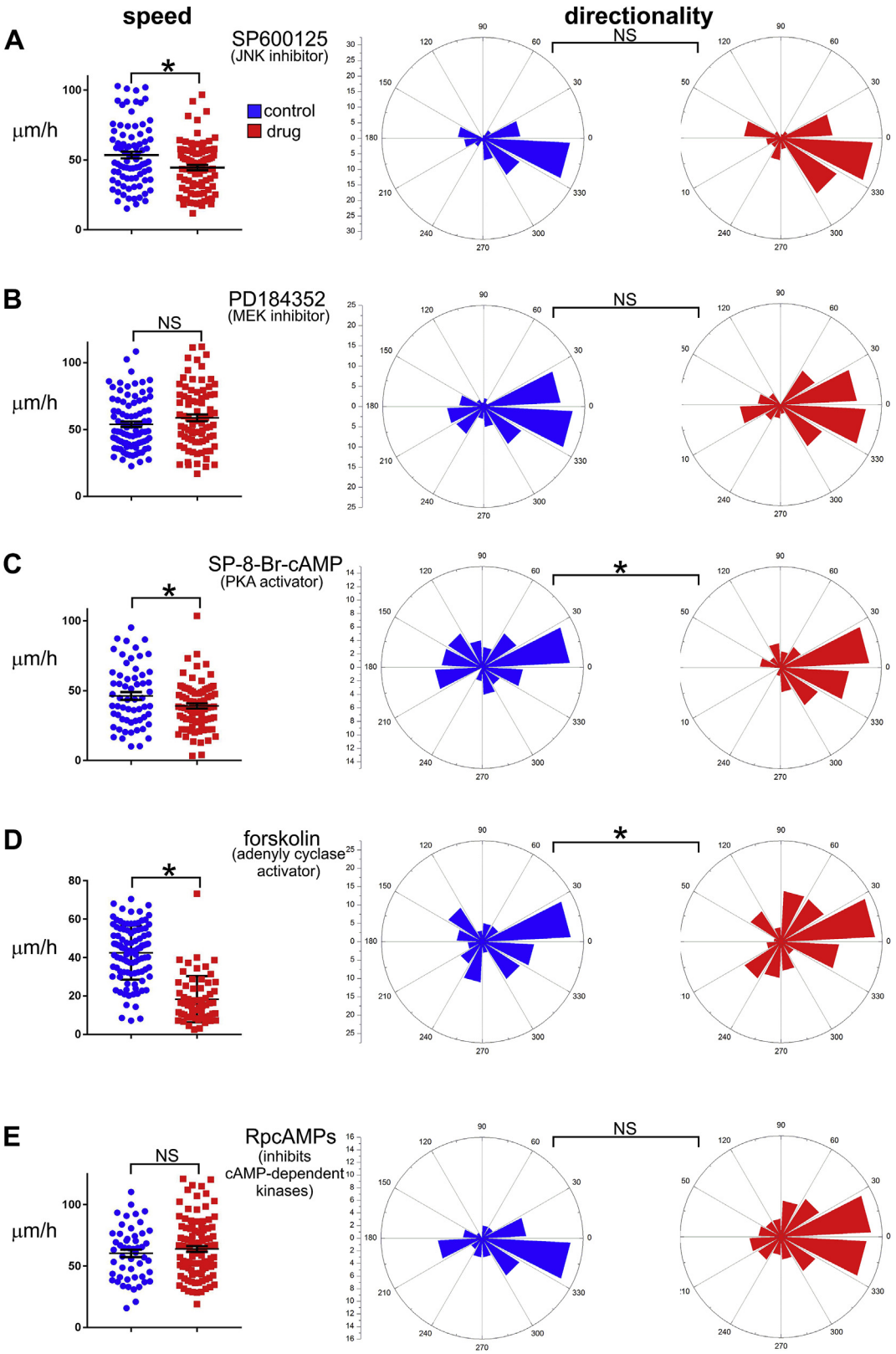

Fig. 2. *Effects of pharmacological perturbations of particular intracellular signalling pathways on speed (LHS) and directionality (polar histograms, RHS) of individual ENCCs in explants of E12.5 colon.* **A.** The JNK inhibitor, SP600125 (30 μM, $n = 8$ experiments), significantly reduced ENCC speed ($^*p = 0.0039$) but had no significant effect on directionality ($p = 0.74$). **B.** The MEK inhibitor, PD184352 (10 μM, $n = 7$ experiments), had no significant effect on ENCC speed ($p = 0.14$) or directionality ($p = 0.55$). **C.** The PKA activator, SP-8-Br-cAMP (120 μM, $n = 5$ experiments), significantly reduced ENCC speed ($^*p = 0.029$) and significantly increased caudal directionality ($^*p = 0.023$). **D.** The adenyly cyclase activator, forskolin (100 μM, $n = 5$ experiments), also significantly reduced ENCC speed ($^*p < 0.0001$) and significantly increased caudal directionality ($^*p = 0.00046$). **E.** However, the cAMP-dependent kinase inhibitor, RpcAMPs (50 μM, $n = 5$ experiments), had no significant effect on ENCC speed ($p = 0.36$) or directionality ($p = 0.06$). Speeds were analysed using unpaired t tests, and directionality by using Watson's U^2 test for angles. Significance was set at 0.05. All experiments were imaged between 4 and 12 h.

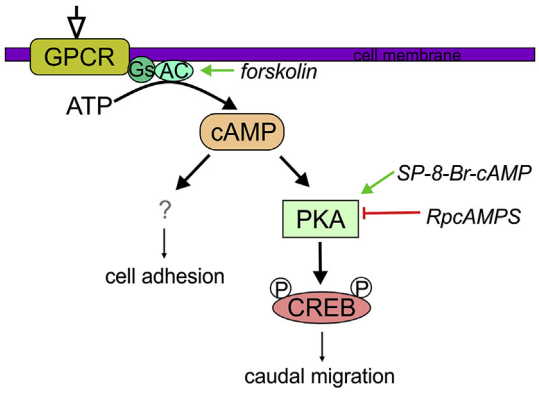

Fig. 3. *Diagram of PKA pathway showing drugs used and a summary of effects on ENCC migratory behaviour and cell-cell adhesion.* As exposure to forskolin, but not SP-8-Br-cAMP, decreased cell-cell adhesion, the effects of forskolin on ENCC adhesion must be independent of PKA. Abbreviations: GPCR (G-protein coupled receptor), AC (adenylyl cyclase), CREB (cAMP response element binding protein).

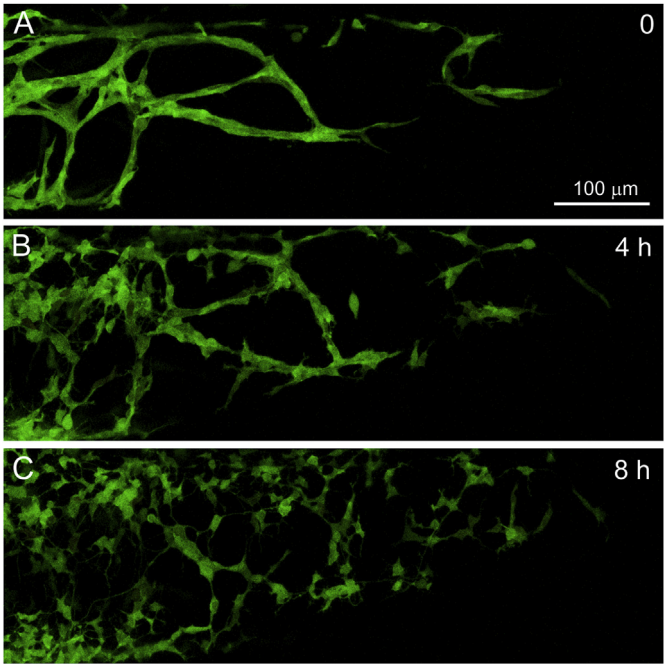

Fig. 4. *Z projections of ENCCs in explants of colon from an E12.5 Ednrb-hKikGR mice showing that the adenyly cyclase activator, forskolin, reduces ENCC cell-cell contact.* **A.** Prior to exposure to forskolin, ENCCs are in chains with high cell-cell contact. 4 h (**B**) and 8 h (**C**) following exposure to forskolin, there is a time-dependent disruption to the chains and a decrease in cell-cell contact.

regulate human mesenchymal stem cell migration and adhesion (Yu et al., 2016), so it would be interesting for future studies to examine the role of Epac in ENCC and other neural crest cell populations as cell-cell adhesion appears to play an important role in the migration of most neural crest cell populations (Szabo and Mayor, 2018).

Activation of the adenylyl cyclase-PKA pathway can lead to the phosphorylation of CREB. Intact gut explants contain a variety of cells types, so to determine if forskolin acts directly on ENCCs, we examined the localization of pCREB using immunohistochemistry. Control segments of small intestine and colon from E12.5 mice, and segments that had been exposed to forskolin for 30 min, were fixed and processed for immunohistochemistry using antibodies to the ENCC-marker, Sox10, and the pan-neuronal marker, Hu. In control preparations, only around 1–2% of both Sox10+ and Hu+ cells in the small intestine, and 10% of Sox10+ and Hu+ cells in the colon, were also pCREB+ (Fig. 5 and Table 1), whereas in forskolin-exposed preparations, around 95% of Sox10+ cells and around 80–90% of Hu+ cells were pCREB+ in both the small intestine and colon. In both control and forskolin-treated preparations, scattered pCREB+ cells that were not KikGR+ and therefore not ENCCs were observed at low density (Fig. 5). These data show that at least some of the effects of forskolin on the migratory behaviour of ENCCs are likely to be direct.

As SP-8-Br-cAMP, a PKA activator, and forskolin, an adenylyl cyclase activator, decreased ENCC speed and increased caudal directionality of ENCCs, we examined the effects of RpcAMPS, which inhibits activation of cAMP-dependent kinases. However, RpcAMPS at 2 different concentrations, 50 μM and 500 μM (Fig. 2E and data not shown), had no significant effect on ENCC speed or directionality. A previous study that used mice expressing FRET biosensors showed that another PKA inhibitor, H89, failed to change PKA activity in ENCC in intact explants of embryonic mouse colon (Goto et al., 2013) and so it appears likely that PKA inhibitors do not permeate through the serosa in intact preparations of embryonic gut.

Both speed and directionality are important for normal ENCC migration (Young et al., 2014). We have demonstrated here that these two migratory characteristics can be differentially affected by perturbing key intracellular signalling pathways in ENCCs. This is not surprising given that ENCCs have intrinsic migratory ability and are also capable of responding to environmental cues that regulate the direction in which they migrate. Pharmacological inhibition of JNK signalling decreased the speed of ENCC migration, but did not impact on ENCC directionality, confirming that ENCC speed and directionality can be unpaired. Although SP600125, the JNK inhibitor used in the current study, has been shown to be a potent and selective inhibitor of JNKs, in future studies it would also be interesting to examine the effects of small molecule JNK inhibitors, or genetically inactivating downstream JNK targets such as c-Jun, on ENCC speed and directionality. Interestingly, activation of PKA with SP-8-Br-cAMP or activation of adenylyl cyclase activity with forskolin resulted in a decrease in migratory speed, but an increase in caudal directionality. Our study raises many questions. For example, it is unclear why slower migration is associated with increased caudal directionality following PKA or adenylyl cyclase activation. It is possible that small differences in cAMP signalling in different subcellular locations within an individual ENCC have a big effect on migration direction. It is unclear which upstream receptors regulate JNK and cAMP activities to regulate speed and directional migration. Although RET and ETB are the best characterized pathways, other receptors can also regulate ENCC speed and direction via JNK or cAMP. Cells exposed to forskolin that migrated directionally, also showed a reduction in their adhesion. Typically, solitary ENCCs show less directed and random migration (Young et al., 2014; Broders-Bondon et al., 2012). However, it is possible that the action of PKA on Cdc42 to induce changes in actin dynamics and subsequently more directional behaviours is responsible for this (Goto et al., 2013; Zhang et al., 2012).

A disadvantage of exposing intact explants of gut to drugs, the approach used in the current study, is that drug-induced changes in ENCC speed or directionality may be indirect. Hence, we cannot rule out the possibility that some of the changes in ENCC speed and directionality we observed were indirect effects. Nevertheless, this is currently the best method for investigating changes in ENCC migratory behaviour as we have previously shown ENCC migration is context dependent (Obermayr et al., 2013). Isolating ENCCs in culture impacts their migratory behaviour and responses to drugs, most likely due to the lack of an extracellular matrix, as well as the absence of other environmental factors. In the case of forskolin, our experiments showed that there are likely to be at least

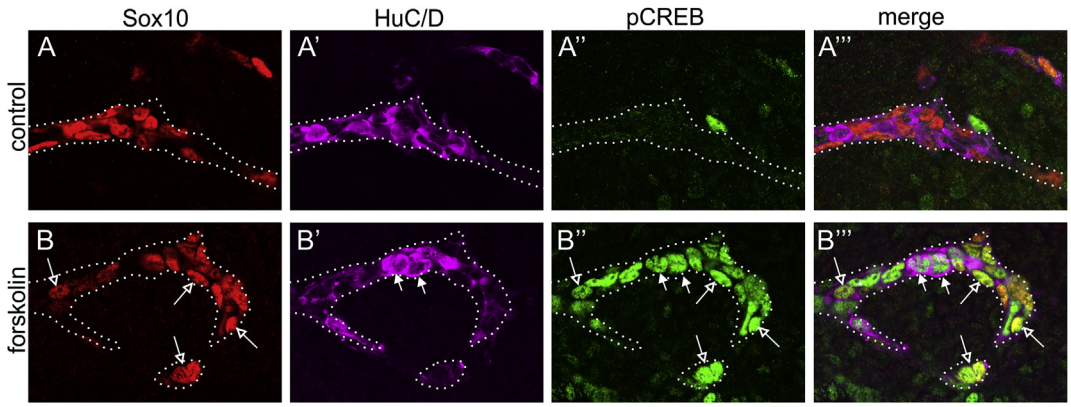

Fig. 5. *Z projections through ENCCs in wholemount preparations of E12.5 proximal colon showing that exposure to forskolin increases the proportion of Sox10+ and HuC/D+ cells showing pCREB immunostaining.* **A.** Group of Sox10+ (A) or HuC/D+ (A′) ENCCs in a control explant of E12.5 colon; none of the ENCCs in this group show detectable pCREB immunostaining (A″), although a non-ENCC just outside the outlined group is pCREB+ (A‴). **B.** Explant of E12.5 colon 30 min following exposure to forskolin (100 μM). Most Sox10+ cells (B, examples shown with open arrows) and HuC/D+ cells (B′, examples shown with closed arrows) show pCREB immunostaining (B″, B‴).

Table 1

Region	Condition	% of Sox10+ cells that were pCREB+	% of Hu+ cells that were pCREB+
Small intestine	control	2% (n = 1224 cells; 3 preparations)	1% (n = 308 cells; 3 preparations)
	forskolin	94% (n = 1649 cells; 3 preparations)	89% (n = 365 cells; 3 preparations)
colon	control	11% (n = 508 cells; 3 preparations)	12% (n = 112 cells; 3 preparations)
	forskolin	95% (n = 879 cells; 3 preparations)	77% (n = 88 cells; 3 preparations)

some direct effects as forskolin induced the expression of pCREB in the vast majority of ENCCs, but in very few non-ENCCs.

In conclusion, our study highlights the complexity of signalling pathways involved in ENCC migratory behaviour and suggest that understanding the pathways involved in directional migration will require imaging of signalling molecule activity at the subcellular level in ENCCs as they migrate and respond to directional cues. As speed and directionality are critical factors for the colonisation of the gut by ENCCs during development, it would be interesting to examine whether mutations in the JNK, PKA and adenylyl cyclase signalling pathways are associated with human enteric neuropathologies, such as Hirschsprung disease, although this would seem unlikely as these pathways are used by most cell types.

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List of Abbreviations

ENCC	enteric neural crest-derived cell
ET-BR	endothelin receptor B
EDN3	endothelin-3

Declarations

Ethics approval and consent to participate

All experiments were approved by the Anatomy & Neuroscience, Pathology, Pharmacology and Physiology Animal Ethics Committee of the University of Melbourne.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

AB, HN, PD, LB, CH and KZ performed experiments and analysed data. MH, HY and LS designed the study, analysed data and wrote the manuscript. All authors read and approved the final manuscript.

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