RESULTS

Effects of chemicals on the migration of cNCCs

Of the 13 chemicals we tested, six chemicals, that is, 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate, significantly inhibited the migration of cNCCs at their embryotoxic concentrations. 13-*cis*-Retinoic acid reduced the migration of cNCCs by approximately 13% at concentrations of 3 and 10 μM (Fig. 3A). Ethanol, ibuprofen, salicylic acid and selenate reduced the migration of cNCCs by 10.5% at 195 mM, 15.9% at 2 mM, 8.5% at 3 mM and 16.2% at 150 μM, respectively (Figs. 3B - E).

Lead acetate reduced the migration of cNCCs by 11.6% at 3 μM and by 30.0% at 10 μM in an initial experiment (Fig. 3F). Because evaluation of the toxic effects of lead at low exposure levels is important for human health, two lower concentrations were added stepwise so that the no-observed-effect level could be estimated. Lead acetate reduced the migration of cNCCs significantly by 8.7% at 1 μM; however, the decrease (6.4%) was not significant at 0.1 μM (Fig. 3G).

The remaining seven chemicals, that is, acetaminophen, caffeine, indium, phenytoin, selenite, tributyltin, and valproic acid, had no significant effects on the migration of cNCCs even at high concentrations (Figs. 4A-G). Indium did not affect the migration of cNCCs and tNCCs in the experiments (Fig. 4C). These experiments for indium were performed at a single concentration for cNCCs and tNCCs because indium showed no effects on the migration of cNCCs in a pilot study and because indium has been reported to cause malformation in the caudal part of rat embryos (Nakajima et al. 2008).

Effects of chemicals on the proliferation of cNCCs

Effects on the proliferation of cNCCs were examined in the case of six chemicals (i.e., 13-*cis*-retinoic acid, ethanol, ibuprofen, lead acetate, salicylic acid, and selenate) that showed inhibitory effects on the migration of cNCCs. The effects of tributyltin on cNCC proliferation were also examined because of our interest in another research project. To reduce the number of animals to be used, two chemicals with the same vehicle were examined concomitantly when possible. In the control groups, the actual cell count increased by approximately 50% during the 24-h exposure period.

13-*cis*-Retinoic acid did not significantly reduce the proliferation of cNCCs at concentrations of 3 and 10 μM, the same concentrations at which it inhibited the migration of cNCCs, although the cell count ratio and the cell proliferation ratio were lowered by 2.5% and 9.7%, respectively, at 3 μM compared to the control group (Fig. 5A).

Ethanol, ibuprofen, salicylic acid, and selenate significantly reduced the cell count ratio by 16.3%, 14.1%, 12.3%, and 20.6%, and the cell proliferation ratio by 59.0%, 43.0%, 33.1%, and 55.4%, respectively, at the same concentrations (195 mM, 2 mM, 3 mM, and 150 μM, respectively) at which they inhibited the migration of cNCCs (Fig. 5B - D).

Lead acetate increased the cell count ratio by 2.2% and the cell proliferation ratio by 6.6% at 1 μM concentration, the lowest effective concentration for inhibiting the migration of cNCCs, although these differences were not statistically significant (Fig. 5C).

Tributyltin reduced the cell count ratio by 9.2% and the cell proliferation ratio by 27.0% at 100 nM concentration, although no reduction in the migration of cNCCs was observed (Fig. 5E).

There was no significant correlation between proliferation inhibition and migration inhibition when the reduced migration was plotted against the reduced cell count ratio, suggesting a varied contribution of the latter to the former (Fig. 6).

DISCUSSION

Here, we observed inhibition of the migration of rat cNCCs by six developmentally toxic chemicals including those not previously reported to have the inhibitory effects: ibuprofen, salicylic acid, and selenate. It is speculated that inhibition of the migration of cNCCs results in reduction of the number of cNCCs at their destination tissues. The inhibited migration of cNCCs by itself, however, seems insufficient as a pathogenic mechanism underlying teratogenicity because these chemicals do not necessarily cause similar malformations. It is probable that the inhibited migration of cNCCs that is not accompanied by an excessive cell shortage is compensated by accelerated cell proliferation at their destination tissues. Alternatively, these inhibitory effects may occur differently in the body of embryos.

From the results of the proliferation assay, it is considered that the reduced cell number may contribute to the inhibited migration of cNCCs to varying extents depending on the test chemicals. It is suggested that the migration-inhibitory effects of ethanol, ibuprofen, and selenate are due in part to the reduced number of cNCCs. In contrast, in the case of tributyltin, the reduced cell number did not affect the migration of cNCCs. Chemicals that did not inhibit cell proliferation, for example, 13-*cis*-retinoic acid, and lead acetate, appeared to inhibit the migration of cNCCs independent of the cell number.

13-*cis*-Retinoic acid appeared to more potently inhibit the migration of cNCCs than all-*trans*-retinoic acid, because the inhibitory concentration of the former (3 μM) was found to be lower than that of the latter (10 μM) in our previous study (Usami et al. 2014b). This is inconsistent with the teratogenic potential of the retinoic acids in rats, where 13-*cis*-retinoic acid is less teratogenic because of its faster elimination from the body (Collins et al. 1994). Isolated cNCCs themselves may be more susceptible to

13-*cis*-retinoic acid than all-*trans*-retinoic acid, as suggested by the lower affinity of 13-*cis*-retinoic acid for cytoplasmic retinoid binding proteins, which may enable easy access to the cell nucleus (Rühl et al. 2001).

Ethanol is a well-known teratogen causing craniofacial malformations (Schardein & Macina 2006) and its toxic effects on NCCs have often been investigated. It was previously shown that ethanol caused apoptotic cell death (Yan et al. 2010) and inhibited migration (Shi et al. 2014) of NCCs. In the present study, both reduced cell number and inhibited migration of cNCCs were observed as the effects of ethanol, although the effective concentration of ethanol was relatively higher than those reported in previous studies, probably because of species and strain differences in the susceptibility to ethanol (Wentzel & Eriksson 2008).

Inhibitory effects of ibuprofen and salicylic acid, which are non-steroidal anti-inflammatory drugs (NSAIDs), on the migration of NCCs have not been reported to date. Although these NSAIDs are considered non-teratogenic in humans, their embryotoxic effects, including craniofacial malformations, observed in animal experiments (Joschko et al. 1993; Kosar 1993) may be related to their migration-inhibitory effects on cNCCs.

The migration-inhibitory effects of lead acetate in the present study are consistent with previously reported results for human NCCs derived from embryonic stem cells (Zimmer et al. 2012). In both studies, lead acetate at 1 μM (20 μg/dl) or higher concentrations inhibited the migration of NCCs without reduced cell proliferation. It is noted that this inhibitory concentration is comparable to blood lead levels (40.0 ± 16.5 μg/dl, mean ± SD) in a certain proportion of pregnant women (Ugwuja et al. 2012). Although lead caused craniofacial malformations only in cultured rat embryos (Zhao et al. 1997)

and does not cause major malformations in humans, its migration-inhibitory effects on cNCCs, as a neuronal progenitor, may be related to functional deficiencies such as neurological alterations (Flora et al. 2011).

The effects of the two selenium compounds on the migration of cNCCs were different in the present study; i.e., selenate inhibited the migration of cNCCs while selenite did not. This difference may be related to the difference in malformed optic vesicles and the protein expression changes caused by the selenium compounds in cultured rat embryos; selenate caused enlargement of the optic vesicle (Usami et al. 2008), a destination of migrating cNCCs (Le Douarin & Kalcheim 1999), and increased the phosphorylated form (inactive form) of cofilin 1 (Usami et al. 2008), an actin-binding protein essential for the migration of NCCs (Gurniak et al. 2005), while selenite did not cause either (Usami et al. 2008). It is thus speculated that selenate inhibits the migration of cNCCs through inactivation of cofilin 1, which results in malformation of the optic vesicle.

In this context, it is intriguing that ethanol and indium also increased phosphorylated cofiline 1 in cultured rat embryos (Usami et al. 2014a; Usami et al. 2009). However, indium did not have inhibitory effects on the migration of cNCCs or tNCCs in the present study. This may indicate that the increase in phosphorylated cofilin 1 alone is not a sufficient condition for inhibition of the migration of NCCs, or that it could occur in different embryonic cells.

The proliferation-inhibitory effects of tributyltin on cNCCs without reduced migration may be related to its developmental toxicity; treatment of pregnant rats with tributyltin that caused blood concentrations comparable to those in the present study, reduced the body weights of pups without causing external malformations (Adeeko et al. 2003; Cooke et al. 2008).

It is unknown at present why the proliferation-inhibitory effects of tributyltin were not accompanied by the inhibited migration of cNCCs. It is unlikely that tributyltin increased the migration of cNCCs, compensating its proliferation-inhibitory effects. This is because tributyltin did not have any effects on the migration of cNCCs over the concentration tested even when the neural tube was removed at 18 h of culture and the cNCCs could move more freely during the exposure period (data not shown). Rather, the relatively selective toxicity and accumulation of tributyltin in the mitochondria (Doherty & Irwin 2011) might have no effects on the migration of cNCCs. In any case, no correlation between proliferation inhibition and migration inhibition means that the NCC migration assay can not be replaced by usual cytotoxicity assays based on the cell number and is valuable to investigate the effects of chemicals on the function of NCCs.

While valproic acid did not inhibit the migration of cNCCs in the present study, the effects of valproic acid on the migration of NCCs are controversial. Valproic acid inhibited the migration of human NCCs in a scratch assay (Zimmer et al. 2012), but did not inhibit the migration of chick NCCs in cultured neural tubes (Fuller et al. 2002). Currently available data indicate that the effects of valproic acid on the migration of NCCs seem to depend on the assay method and the species used in which it is used.

For other chemicals (acetaminophen, caffeine, and phenytoin) that did not inhibit the migration of cNCCs, no particular information concerning the involvement of NCCs' malfunction in their developmental toxicity was found, except that acetaminophen did not inhibit the migration of human NCCs either (Zimmer et al. 2012).

In conclusion, it was established that several developmentally toxic chemicals inhibit the migration of cNCCs, which appears differently as craniofacial abnormalities. Mechanistic investigation is needed to understand the variability in the outcomes of the

inhibited migration of cNCCs. Our migration assay method will be useful for this purpose because of its simplicity.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDGEMENTS

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Legends to the figures

Fig. 1. Culture schedule of for the migration and proliferation assays of cephalic neural crest cells (cNCCs)

Neural tubes were excised from the rhombencephalic region of day 10.5 rat embryos and cultured for 48 h to allow the emigration of cNCCs. Chemicals were added to the culture medium at 24 h. In the proliferation assay, the neural tubes were removed from the culture dishes at 18h leaving the cNCCs behind, and the cell nuclei were fluorescently stained before the photography at 48 h.

Fig. 2. Photographs of cephalic neural crest cells (cNCCs) cultured in the migration and proliferation assays

(A) cNCCs cultured in the migration assay are shown with blue polygons connecting the outermost cells for the calculation of the cell migration. (B) cNCCs cultured in the proliferation assay are shown with blue dots (24 h) or stained cell nuclei (48 h) for the determination of the cell count.

Fig. 3. Migration of cephalic neural crest cells (cNCCs) cultured in the presence of developmentally toxic chemicals with migration-inhibitory effects

Migration indices were calculated as the radius ratio from the circular spread of cNCCs at 24 and 48 h of culture. The mean ± standard error of the mean (SEM) values of 6–27 neural tubes are shown. Asterisks indicate statistically significant differences from the corresponding control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$).

Fig. 4. Migration of cephalic neural crest cells (cNCCs) cultured in the presence of

developmentally toxic chemicals without migration-inhibitory effects

Migration indices were calculated as the radius ratio from the circular spread of cNCCs at 24 and 48 h of culture. The mean ± standard error of the mean (SEM) values of 8–16 neural tubes are shown. Effects of indium were examined also in trunk neural crest cells as shown in (C).

Fig. 5. Proliferation of cephalic neural crest cells (cNCCs) cultured in the presence of developmentally toxic chemicals

The cNCCs were counted at 24 and 48h of culture, and the proliferation indices were calculated. The mean ± standard error of the mean (SEM) values of 7–10 neural tubes are shown. Asterisks indicate statistically significant differences from the corresponding control (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Fig. 6. Plot of the reduced migration versus the reduced cell count ratio of neural crest cells cultured in the presence of developmentally toxic chemicals

The reduced migration and reduced cell count ratio were calculated by subtracting the corresponding data in Figs. 1–3 from 100%. The linear regression line and correlation coefficient (r) for all the plotted data are shown.

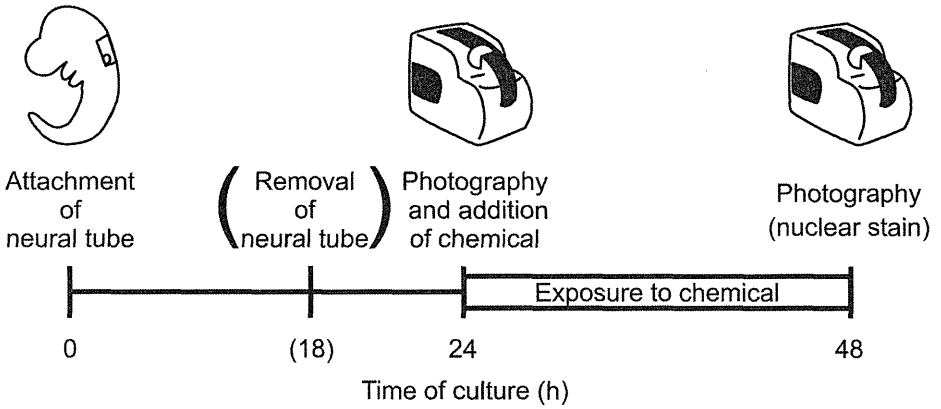

Fig. 1

CGA_12121_F1

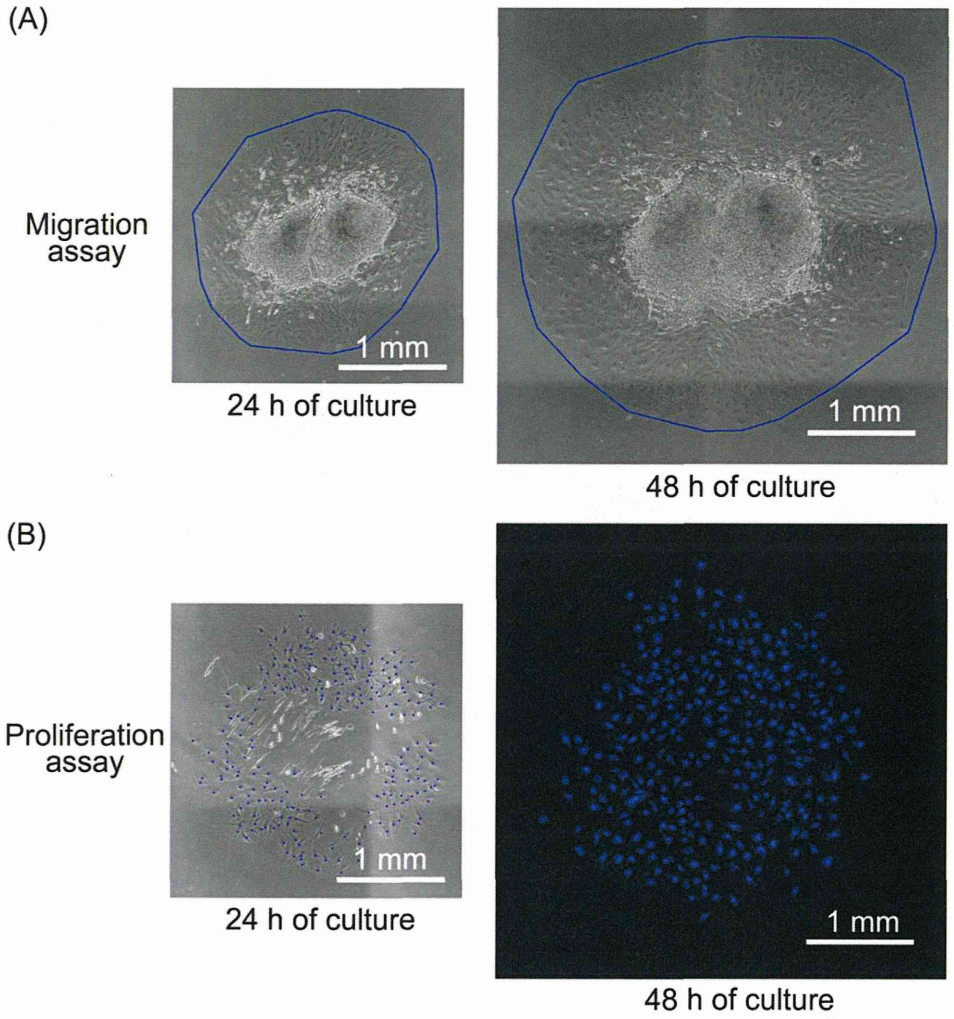

Fig.2

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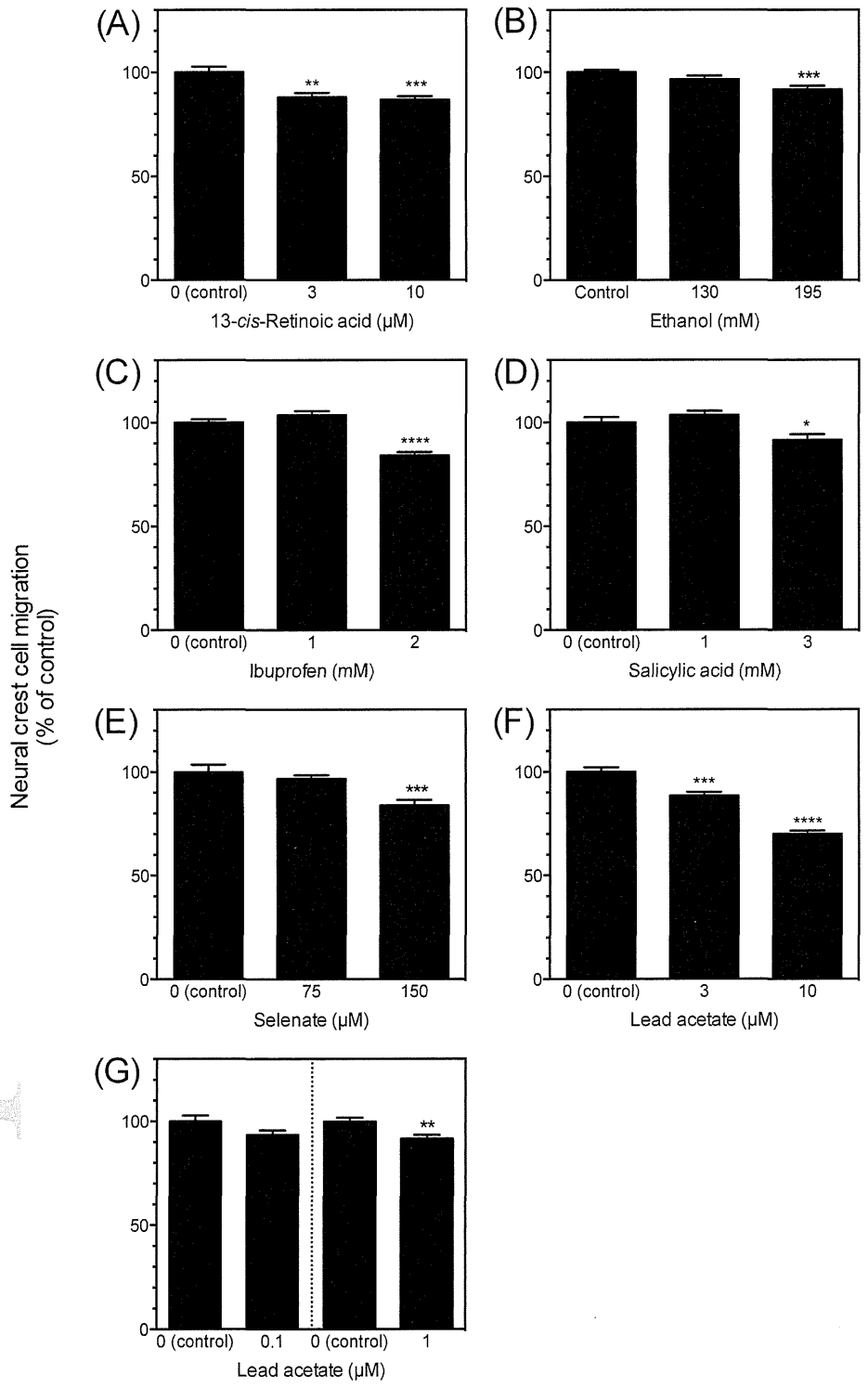

Fig.3

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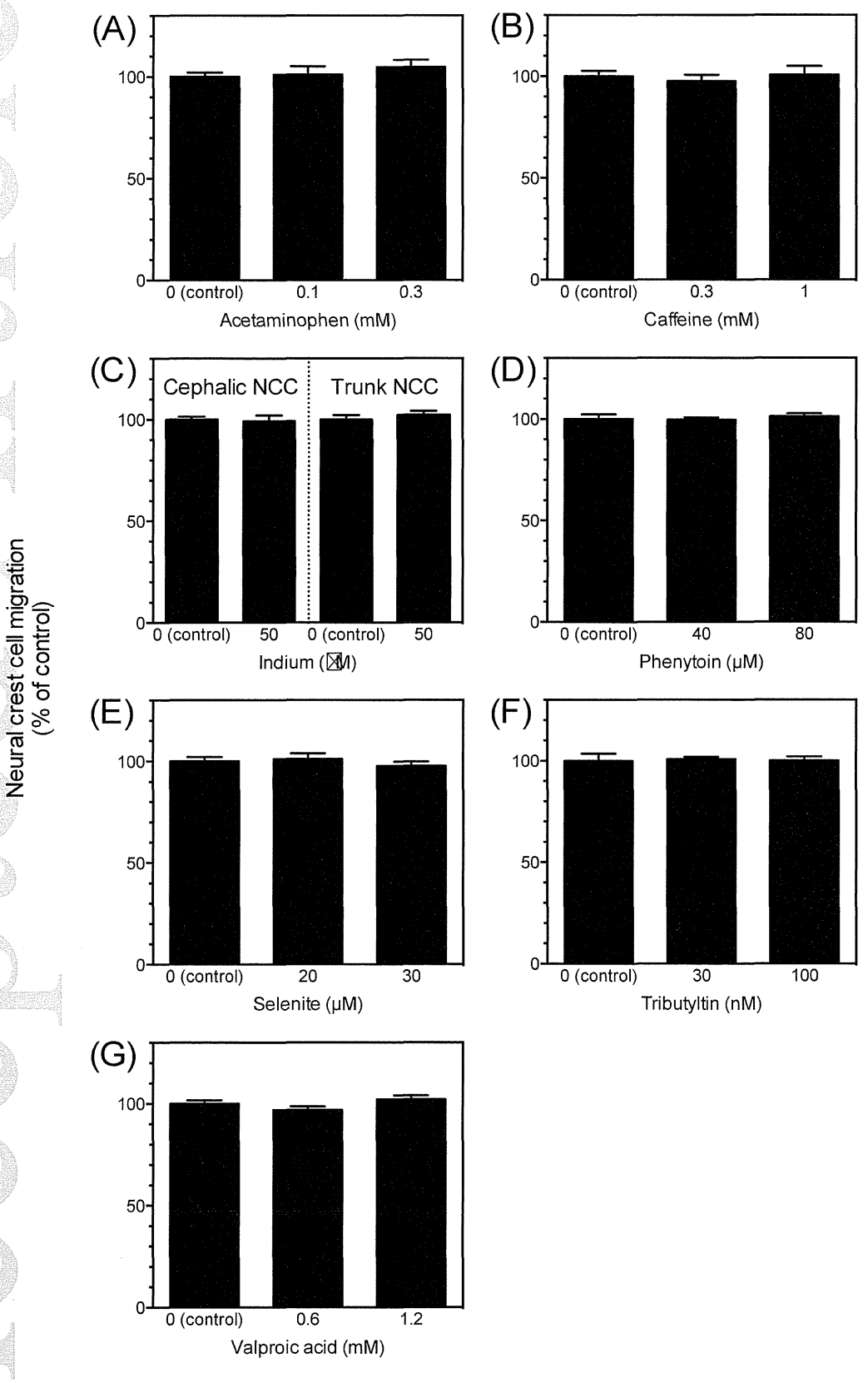

Fig.4

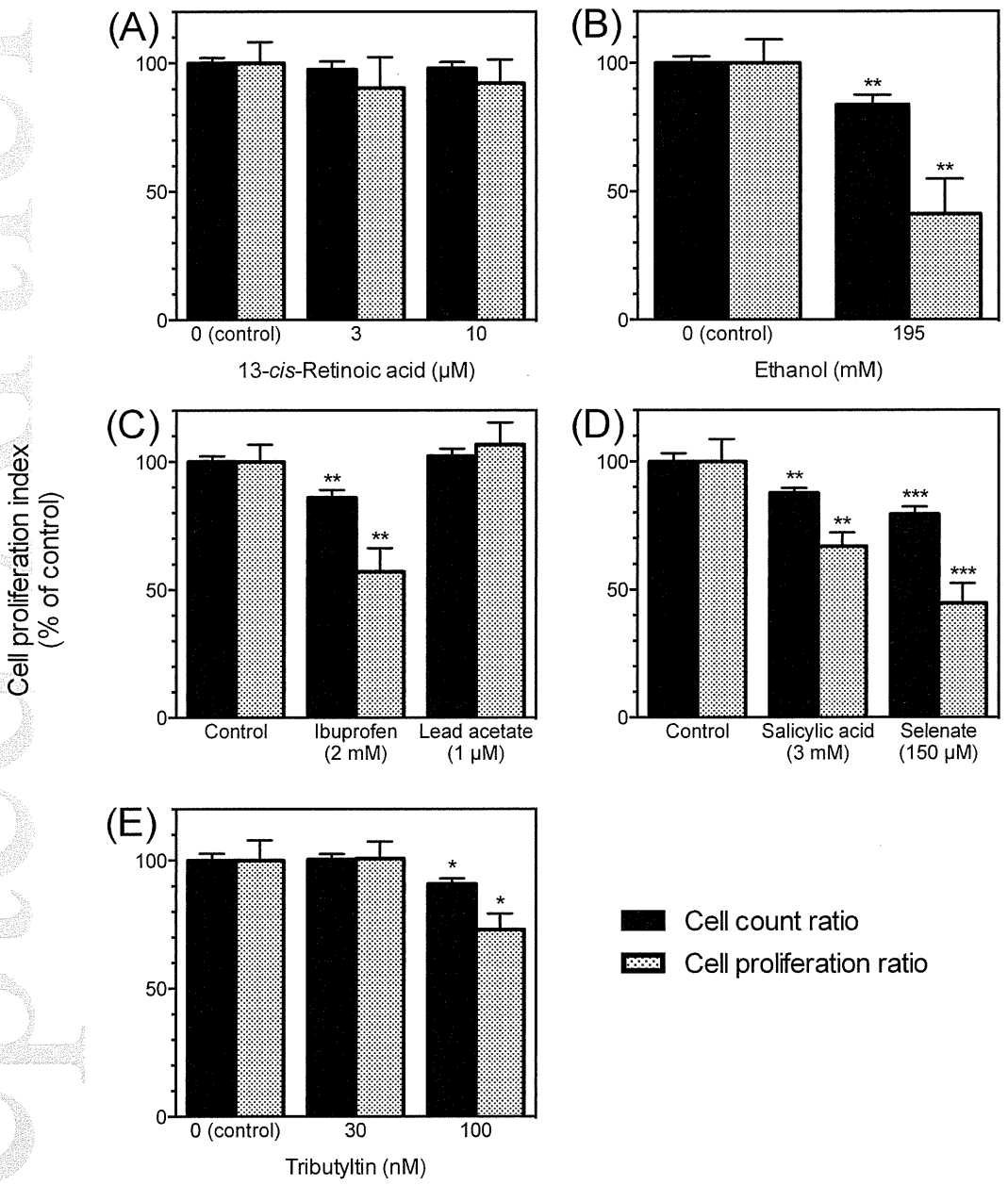

Fig.5

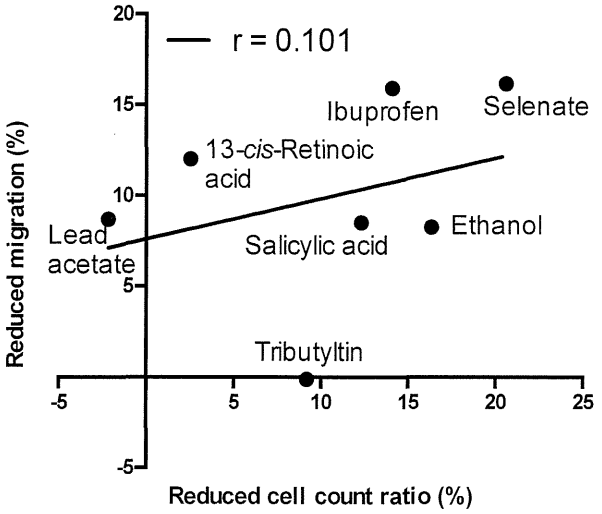

Fig.6

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