

# Estrogen Promotes the Initial Migration and Inception of NgCAM-Dependent Calcium-Signaling by New Neurons of the Adult Songbird Brain

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The adult avian forebrain continues to generate neurons from ventricular zone (VZ) precursor cells, whose neuronal progeny then migrate into the brain parenchyma. Migrating neurons respond to the Ig-family adhesion molecule NgCAM with increments in cytosolic calcium, and migration is disrupted by anti-NgCAM Ig. The calcium response to NgCAM is developmentally restricted to bipolar migrants during a period spanning 6 to 9 DIV. This period corresponds to the postmitotic age at which new neurons leave the adult VZ to traverse a subjacent layer of estrogen-receptive “gatekeeper” neurons. Since neuronal passage through this layer occurs concurrently with the onset of NgCAM-dependent calcium signaling, we asked whether acquisition of the calcium response to NgCAM required estrogen exposure. Among neurons arising from explants of the adult finch neostriatal VZ, only those supplemented with estrogen developed calcium responses to NgCAM; neither explants raised in the absence of estrogen, nor those supplemented with testosterone, did so. Neurons in all three groups expressed NgCAM, had equivalent baseline calcium levels, and responded identically to K<sup>+</sup>-depolarization. Nonetheless, many more neurons migrated from explants of both finch and canary VZ raised in estrogen-supplemented media than from their estrogen-deprived counterparts, even though no effect of estrogen on neuronal survival per se was noted. These findings suggest that estrogen encourages the initial departure and assumption of signal competence by neurons arising from the adult avian VZ, thereby promoting their parenchymal recruitment and migration success.

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## INTRODUCTION

The adult avian forebrain continues to generate neurons from ventricular zone (VZ) precursor cells (Goldman and Nottebohm, 1983), whose neuronal progeny migrate into the brain parenchyma along ependymally derived radial guide fibers (Alvarez-Buylla and Nottebohm, 1988; Goldman *et al.*, 1993). The radial guide cells, like their neuronal partners, arise through *in situ* division (Alvarez-Buylla *et al.*, 1990), and both cell types can arise from a common pluripotent precursor (Goldman *et al.*, 1996b), just as in early embryogeny (Gray and Sanes, 1992). However, unlike the rapid departure of new neurons observed from embryonic neuroepithelium, the newly produced neurons of the adult avian brain persist in the VZ for at least 4 days after their parental precursor cell division (Barami *et al.*, 1995). They leave the VZ only after down-regulating the surface adhesion molecule N-cadherin, which is otherwise tonically expressed by all VZ cells, including neuronal precursors (Barami *et al.*, 1994). Upon departing the VZ, new neurons express the immunoglobulin-family adhesion molecule NgCAM (Grumet *et al.*, 1983). Anti-NgCAM Fab fragments markedly disrupted the *in vitro* migration of these cells (Barami *et al.*, 1994), suggesting that NgCAM function is required for the progression of neuronal migration from the adult VZ (reviewed in Goldman, 1998; Goldman and Luskin, 1998).

The disruption in migration appeared similar to that experienced by migrating embryonic cerebellar granule neurons in response to antibodies against the closely related NgCAM-family member, L1 (Grumet *et al.*, 1983;

Lindner *et al.*, 1983; Lindner *et al.*, 1986). However, migrating adult VZ-derived neurons died prematurely after exposure to anti-NgCAM, suggesting that NgCAM's role in this system might extend to include the support of early neuronal survival. To investigate this possibility, we used confocal imaging of cultures of the finch VZ loaded with the calcium indicator fluo-3, to test the effect of NgCAM on calcium-mediated signal transduction in newly generated adult neurons. We found that new neurons responded to NgCAM protein with reversible increments in cytosolic calcium. These calcium responses to NgCAM were G-protein dependent and mediated through voltage-gated calcium channels (Goldman *et al.*, 1996a). Similar NgCAM-triggered calcium signaling had been noted previously in both PC12 (Schuch *et al.*, 1989; Von Bohlen und Halbach *et al.*, 1992) and primary embryonic neurons (Walsh and Doherty, 1992; Williams *et al.*, 1992, 1994). However, the NgCAM-driven calcium responses of new neurons in the adult songbird brain were noted for only a restricted period of migration, despite the persistent and stable expression of NgCAM by these cells (Goldman *et al.*, 1996a). This transient period of NgCAM signal competence, which spanned 6–9 days *in vitro*, corresponded to the postmitotic age at which new neurons left the adult VZ to enter the parenchyma *in vivo* (Barami *et al.*, 1995). Accordingly, the calcium response to NgCAM was essentially limited to migrating bipolar cells (Goldman *et al.*, 1996a).

On the basis of these studies, we asked whether the coupling of NgCAM expression to NgCAM-dependent calcium signaling was cell-autonomous, or whether it was instead regulated by the environment of the cells during their initial migration. The latter possibility was suggested by the observations that (1), during the initial migration of new neurons from the adult avian VZ, they traverse a dense layer of subventricular neurons that express estrogen receptor protein (ER), and (2), estrogen substantially increases the number of neurons recruited into the adult mediocaudal neostriatum (Hidalgo *et al.*, 1995). These findings suggested that ER<sup>+</sup> subventricular cells might regulate the initial departure or survival of those migrants traversing it, in an estrogen-dependent fashion.

Since neuronal migration *in vitro* was associated with the appearance of a transient calcium response to exogenous NgCAM, and since the time course of this response corresponded roughly to that of neuronal transit through the estrogen-receptive zone *in vivo*, we postulated that the development of NgCAM-related calcium signaling might depend on antecedent estrogen exposure. In testing this hypothesis, we observed that

estrogen treatment was associated with a substantial increase in the number of neurons migrating from explants of both adult zebra finch and canary HVC. These neurons indeed exhibited robust calcium responses to NgCAM, in contrast to their untreated counterparts, which were generally unresponsive to NgCAM. Thus, estrogen promoted neuronal differentiation and/or departure from the adult songbird VZ, while inducing or permitting the new migrants to couple NgCAM expression to CAM-dependent signaling cascades important to the progression of migration.

## RESULTS

### *Estrogen Regulates NgCAM-Mediated Signaling by New Neurons*

We first asked whether the passage of new neurons through the estrogen-receptive layer and their development of NgCAM-related calcium signaling were causally related. To that end, we raised adult neostriatal VZ explants in steroid-depleted medium (SDM) alone, or in SDM supplemented with either estrogen or testosterone. We then compared the development of calcium signaling to NgCAM in each treatment group.

Explants of the neostriatal VZ, including that overlying the highly neurogenic vocal control nucleus HVC, were prepared from adult female zebra finches as previously described (Goldman, 1990; Goldman and Nedergaard, 1992; Goldman *et al.*, 1992). The VZ explants were raised in a steroid-depleted base medium, composed of phenol red-free DMEM/F12/N2 (Bottenstein and Sato, 1979), containing charcoal-stripped fetal bovine and capon sera, each at 10% v/v, and further supplemented with nonessential amino acids, fatty acid-rich albumin, progesterone, hydrocortisone, and triiodothyronine (see Experimental Procedures). This base medium has undetectable levels of estrogen or testosterone (Hidalgo *et al.*, 1995). The test media were composed of this base, to which estradiol (1 ng/ml), testosterone (10 ng/ml), or cholesterol (10 ng/ml) were added. By 5 DIV, productive explants generated an outgrowth of initially bipolar postmitotic neurons, which matured morphologically over the 2–3 days thereafter. At 7 DIV, test neuronal outgrowths were loaded with the calcium-sensitive dye fluo-3, then exposed to purified chicken NgCAM (1.2 µg/ml) with concurrent observation by confocal microscopy. Cultures were tested at 7 DIV since in our initial description of NgCAM-dependent calcium signaling in this system, performed in medium containing whole serum with high levels of gonadal steroids,

the calcium increment to NgCAM was greatest at 7 DIV (Goldman *et al.*, 1996a).

We found that in cultures raised in estrogen-supplemented media, neurons responded to NgCAM with an average maximum calcium elevation of  $82 \pm 21\%$  within an hour after NgCAM addition (mean  $\pm$  SD;  $n = 110$  neurons) (Figs. 1 and 2). This rise was significantly greater than that mounted over the same time span by their counterparts raised in estrogen-deficient media, which exhibited little calcium response to NgCAM ( $13 \pm 8\%$ ,  $n = 222$ ). The difference in the neuronal calcium response to NgCAM between estrogen-treated and cholesterol control cultures, was  $P < 0.01$  by 2-way ANOVA (Table 1).

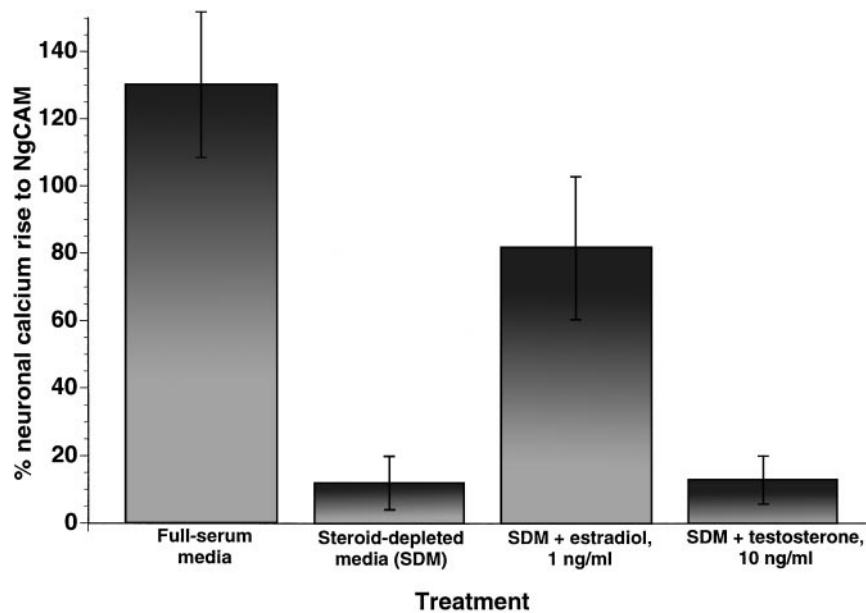
### Testosterone Failed to Permit NgCAM-Mediated Signaling by New Neurons

The *in vivo* recruitment and survival of newly generated neurons in the adult songbird brain can be influenced by androgens as well as estrogens (Rasika *et al.*, 1994). We therefore also examined the effect of testosterone on the development of NgCAM-dependent calcium signaling by new neurons. Adult finch VZ explants were raised in steroid-depleted media to which 10 ng/ml of

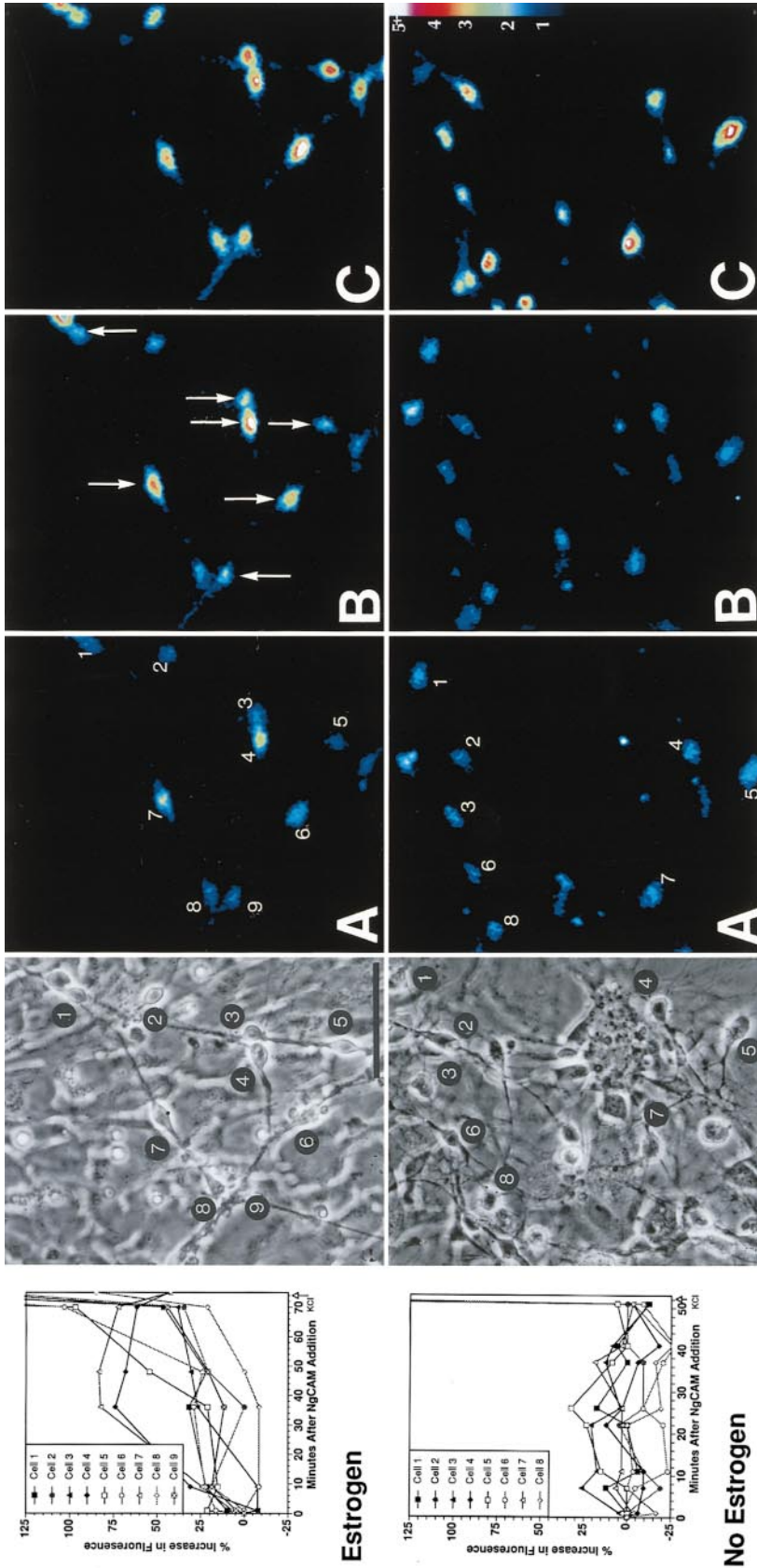
testosterone was added. We found that this testosterone exposure was not associated with the development of calcium signaling to NgCAM; cultures supplemented with testosterone exhibited a mean calcium increment to NgCAM of  $13 \pm 7\%$  ( $n = 148$ ), which was no different from the response displayed by explants raised in steroid-deficient media alone ( $13 \pm 8\%$ ,  $n = 222$ ) (Fig. 3). Thus, the coupling of NgCAM to its dependent calcium signaling pathways was effected by exposure to estrogen, but not androgen (Table 1).

### Estrogen Did Not Influence Neuronal Calcium Levels or Responses to Depolarization

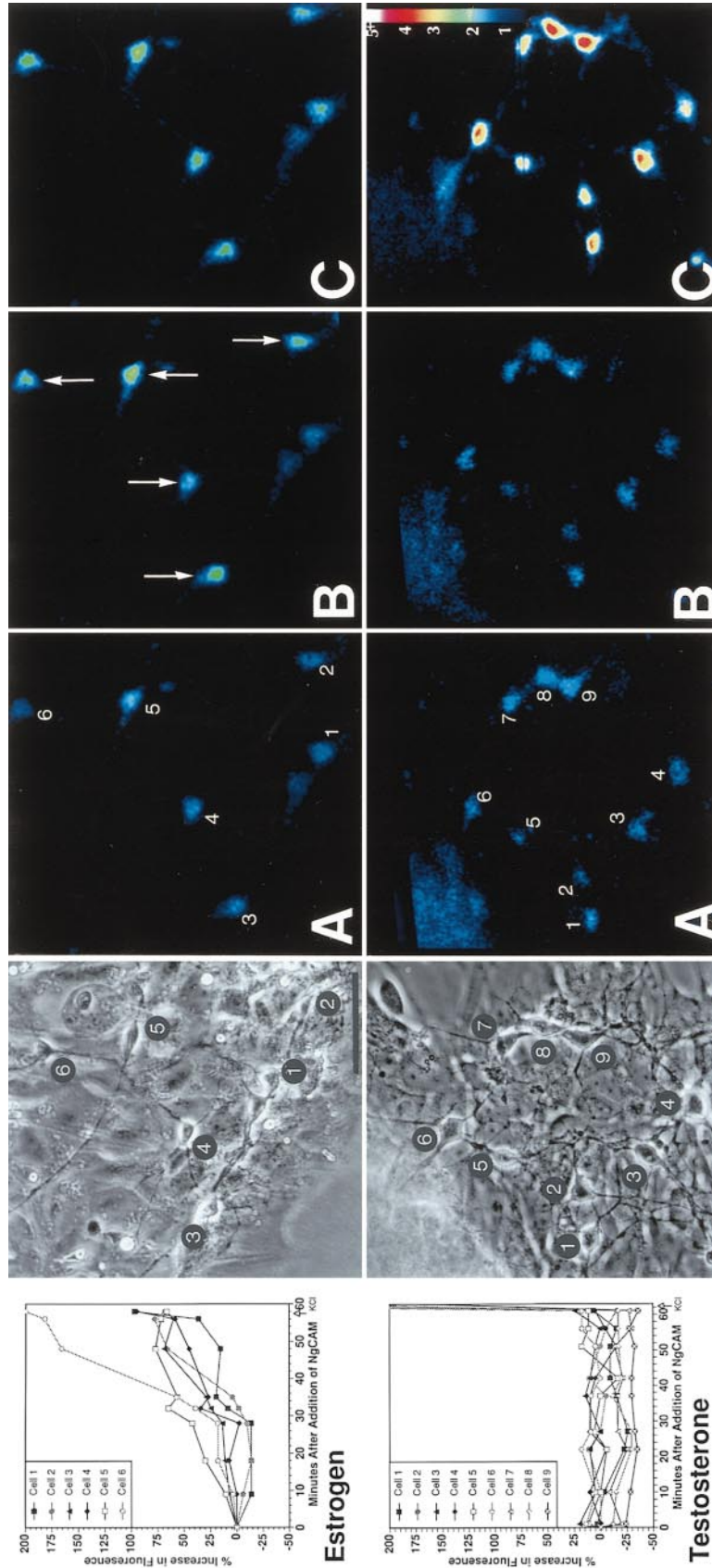
The differences in neuronal calcium responses to NgCAM between estrogen-deficient and estrogen-exposed neurons did not result from any effect of estradiol on either baseline calcium, or on the density or activity of voltage-gated calcium channels: Neurons raised in steroid-depleted and estrogen-supplemented media had nearly identical baseline concentrations of cytosolic calcium, as determined by fura-2 imaging (Table 2). Furthermore, neurons in each treatment group developed similar responses to  $K^+$ -depolarization: Fluo-3 imaging by confocal microscopy revealed that at 7–8



**FIG. 1.** Calcium increments to NgCAM were exhibited only by new neurons raised in the presence of estrogen. This graph plots the average NgCAM-triggered rise in  $Ca^{2+}$ , reflected by the fluo-3 fluorescence signal, of neurons generated from explants of the adult zebra finch VZ, as a function of gonadal steroid exposure. NgCAM-driven neuronal calcium responses were observed to develop in estrogen-containing media, whether the steroid was already present in whole serum or added to steroid-depleted media (SDM). Among 7 DIV explants raised in steroid-depleted media, only those neurons exposed to added estrogen exhibited significant calcium responses to NgCAM ( $P < 0.01$  by ANOVA). Growth in testosterone-supplemented media failed to permit NgCAM-dependent calcium signaling.



**FIG. 2.** Estrogen was required for development of neuronal calcium responses to NgCAM. These two rows compare the calcium responses of adult finch VZ-derived neurons to NgCAM (1.2  $\mu\text{g}/\text{ml}$ ), in estrogen-supplemented (top row) and -deficient (bottom row) media. These sister cultures were both tested at 7 DIV. NgCAM activated a neuronal calcium response among neurons raised in estradiol-supplemented media, but not in matched unsupplemented cultures. In this plate, the left-hand panel graphs the  $\text{Ca}^{2+}$  levels of each neuron in the adjacent phase micrograph, as a function of time. A represents the calcium image of each fluo-3 preloaded field 15 min after achieving a stable baseline, just before NgCAM addition. B shows the maximal neuronal calcium response to NgCAM, achieved within the first 45 min after NgCAM addition. C shows the response of the recorded neurons to potassium depolarization, 1 h after NgCAM exposure. Whereas neurons raised in estrogen-deficient media showed little  $\text{Ca}^{2+}$  rise to NgCAM, most of the neurons in the matched, estradiol-supplemented culture responded significantly (arrows). In both treatment groups, neurons responded to potassium-depolarization with rapid influxes of calcium.



**FIG. 3.** Testosterone treatment, unlike estrogen, did not permit the development of the neuronal calcium response to NgCAM. NgCAM failed to evoke a neuronal calcium response among neurons raised in testosterone-supplemented media, even though it stimulated substantial calcium increments in estrogen-exposed plates. These two rows examine the calcium responses of new neurons in 7 DIV adult finch VZ cultures to NgCAM (1.2  $\mu\text{g/ml}$ ). The top row shows the NgCAM-stimulated calcium responses of neurons raised in estrogen-supplemented media. Their counterparts (bottom row) raised in testosterone-supplemented media failed to exhibit any calcium response to NgCAM media. As in Fig. 2, the left-hand panel of each row graphs the  $\text{Ca}_i^{2+}$  level of each imaged neuron as a function of time. A shows the fluo-3 preloaded field 15 min after achieving a stable baseline, just before NgCAM addition. B shows the maximal neuronal calcium response to NgCAM, here at 45 min after NgCAM addition. C shows the subsequent neuronal response to 60 mM KCl. Whereas neurons raised in testosterone-treated cultures (bottom row) showed little  $\text{Ca}_i^{2+}$  rise to NgCAM, most estrogen-exposed neurons responded significantly (top row, arrows). Neurons in both groups responded to KCl-depolarization with roughly equivalent influxes of calcium.

**TABLE 1**  
Estrogen Permitted NgCAM-Dependent  $\text{Ca}_i^{2+}$  Signaling by Adult VZ-Derived Neurons

Media	Treatment	Cells counted <sup>a</sup>	Maximum Ca rise (%) <sup>b</sup>	Maximum Ca rise to KCl
Steroid-depleted media (SDM)	NgCAM, 1.2 $\mu\text{g}$	222	13 $\pm$ 8	242 $\pm$ 25%
SDM + estradiol <sup>c</sup>	NgCAM	110	82 $\pm$ 21*	191 $\pm$ 39%
SDM + testosterone <sup>d</sup>	NgCAM	148	13 $\pm$ 7	253 $\pm$ 26%

<sup>a</sup>Cultures of the adult female zebra finch neostriatal VZ were tested at 7 or 8 DIV. Only those neuron-like cells with Ca increments  $\geq$ 100% to 60 mM KCl were included in the sample, so as to ensure both the neuronal identity and viability of the cells tested.

<sup>b</sup>The maximum neuronal calcium level achieved within the first hour after NgCAM exposure. Means  $\pm$  standard deviation.

<sup>c</sup>Estradiol at 1 ng/ml.

<sup>d</sup>Testosterone at 10 ng/ml.

\* $P < 0.01$  by 1-way ANOVA.

DIV, by which time NgCAM effects are most pronounced, neurons responded to  $\text{K}^+$ -depolarization with analogous calcium increments in the estradiol-treated (191  $\pm$  39%), testosterone-treated (253  $\pm$  26%), and steroid-depleted control cultures (242  $\pm$  25%) (Table 1).

To ensure that the depolarization-induced changes in relative calcium signals detected by fluo-3 accurately reflected changes in absolute cytosolic calcium, we also used ratio imaging to quantify the depolarization-induced calcium responses of neurons raised in either SDM or SDM supplemented with estradiol. Ratio imaging using fura-2 allows absolute calcium concentrations to be determined. We employed it here after 12 DIV, to

**TABLE 2**  
Estrogen Influenced Neither Baseline Nor Depolarization-Induced Calcium Levels in New Neurons Arising from the Adult Finch Ventricular Zone

	Baseline cytosolic $\text{Ca}_i^{2+}$ (nM)		Depolarization-induced $\text{Ca}_i^{2+}$	
	Estrogen	Control	Estrogen	Control
5 DIV	68.4 $\pm$ 18 <i>n</i> = 54	66.0 $\pm$ 21 <i>n</i> = 57	NA	NA
7 DIV	65.4 $\pm$ 23 <i>n</i> = 71	67.8 $\pm$ 37 <i>n</i> = 71	NA	NA
12 DIV	54.2 $\pm$ 22 <i>n</i> = 25	55.7 $\pm$ 22 <i>n</i> = 16	321.5 $\pm$ 109 <i>n</i> = 25	327 $\pm$ 121 <i>n</i> = 16

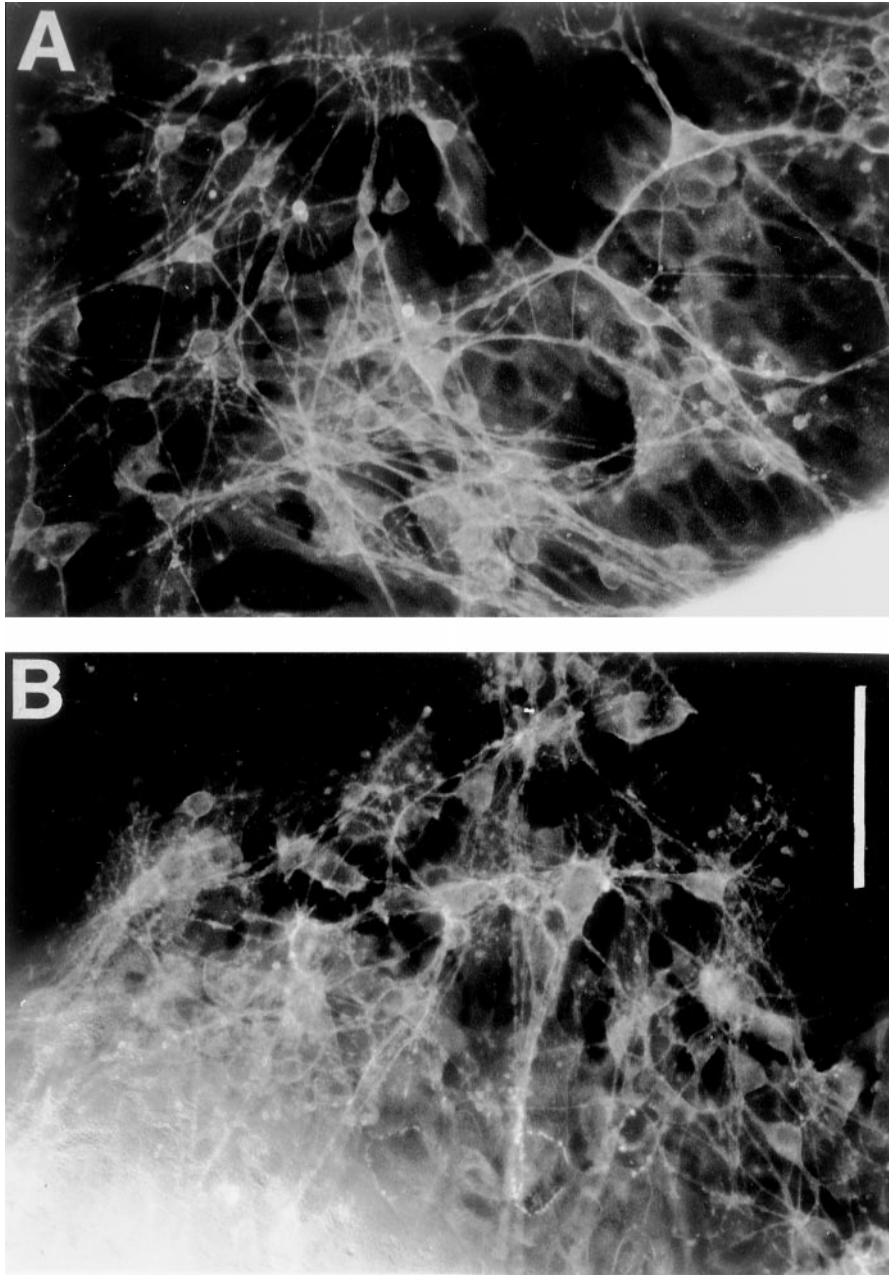
*Note.* Absolute calcium concentrations, as determined by fura-2 imaging. All values as mean  $\pm$  SD. *n* = number of neurons imaged in each group.

allow time for any treatment-related differences in depolarization-induced calcium elevations to develop. We found no difference between estrogen-treated and control neurons in either their baseline or KCl-evoked calcium levels at 12 DIV (Table 2); in each group, depolarization resulted within 10 s in roughly a sixfold increase in cell calcium, starting from equivalent baseline calcium levels (54.2  $\pm$  22 nM  $\text{Ca}_i^{2+}$  among estrogen-exposed neurons, and 55.7  $\pm$  22 nM for steroid-depleted controls), and rising to 320–330 nM  $\text{Ca}_i^{2+}$  within 10 s of depolarization in both estrogen-treated and control cultures (Table 2). Thus, estrogen had no apparent effect on either baseline or depolarization-induced calcium levels in these neurons. This suggested that the estrogenic induction of NgCAM-associated calcium signaling resulted from the specific coupling of NgCAM to its dependent signaling pathways and not from any estrogen-associated changes in calcium homeostasis (Tables 1 and 2).

### **Estrogen and Testosterone Each Increased the Number of Neurons Arising from Adult VZ Explants, without Influencing Their Longer-Term Survival**

Both estrogen (Hidalgo *et al.*, 1995) and testosterone (Rasika *et al.*, 1994) support neuronal production in the adult songbird HVC. Estrogen also promotes the recruitment of new neurons into developing and juvenile vocal control nuclei (Nordeen and Nordeen, 1989). We therefore asked whether estrogen might promote the recruitment of neurons from cultured explants of the adult HVC. To this end, both the outgrowth and survival of neurons arising from adult HVC explants were compared between controls raised in steroid-depleted media and experimentals supplemented with either estrogen or testosterone. In addition, to account for potential species differences between zebra finches, the model system in which our NgCAM-Ca signaling studies were conducted, and canaries, related oscines in which most studies of gonadal steroid effects on neurogenesis have been performed (Brown *et al.*, 1993; Hidalgo *et al.*, 1995; Rasika *et al.*, 1994), we assessed the role of estrogen and testosterone on neuronal outgrowth in each. A total of nine canaries and four finches were dedicated to this experiment, all adult females.

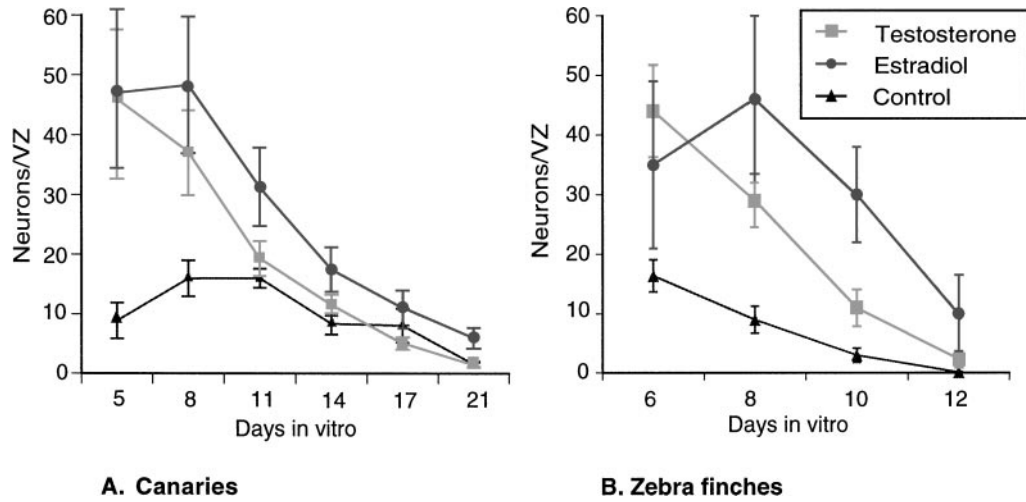
HVC explants derived from both finches and canaries exhibited maximal neuronal outgrowth by 8 DIV, regardless of treatment condition. By that point, neurons in each treatment group expressed similar distributions and intensities of surface NgCAM-immunoreactivity (Fig. 4). In both species, the number of neurons observed during the 5–11 DIV period was significantly higher in



**FIG. 4.** Neurons in estrogen-exposed and -deficient media expressed a similar distribution and intensity of NgCAM immunofluorescence. This plate shows the cellular outgrowth of ventricular zone cultures raised in either (A) gonadal steroid-depleted control media or (B) the same media supplemented with 1 ng/ml estradiol, immunostained for NgCAM after 7 DIV. Neuronal expression of NgCAM-immunoreactivity did not noticeably differ between the two groups. Thus, the divergent effects of NgCAM on calcium levels in new neurons raised in estrogen-exposed and -deficient media did not derive from any effect of estrogen upon neuronal NgCAM expression. Scale, 25  $\mu$ m.

both the estradiol and testosterone-treated explants than in their counterparts exposed to cholesterol (Fig. 5). However, during the 8–12 DIV period thereafter, the slopes of decline in neuronal counts did not differ between these treatments. Indeed, despite the initial

promotion of neuronal outgrowth noted in estrogen-treated cultures, by 12 DIV the number of neurons in the estrogen-containing group was not significantly higher than that in the steroid-depleted controls. Thus, neither estradiol nor testosterone appeared to impart any long-



**FIG. 5.** These graphs plot the mean number of neurons per explant outgrowth as a function of the number of days in culture, compared among explants raised in base media (control), testosterone (10 ng/ml), or estradiol (1 ng/ml). (A) Canaries. Neuronal outgrowth from explants of adult canary VZ was increased substantially by both testosterone and estradiol. Yet by 2 weeks *in vitro*, neuronal loss was substantial in both groups, such that no long-term survival effect was noted to gonadal steroid treatment. (B) Finches. Just as in cultures of the canary neostriatal VZ, significantly greater numbers of neurons emigrated from adult finch VZ explants in estrogen- and testosterone-supplemented media than in their cholesterol-supplemented controls. The zebra finch cultures were assessed over a shorter time frame (6–12 DIV) than their canary counterparts (5–21 DIV).

term survival benefit to neurons once they appeared in the explant outgrowth. Notably, previous studies had demonstrated that neither estrogens nor androgens influence neuronal mitogenesis in this preparation, despite their mutual promotion of postmitotic neuronal recruitment *in vivo* (Brown *et al.*, 1993; Hidalgo *et al.*, 1995; Rasika *et al.*, 1994). Thus, estrogen appeared to promote either the postmitotic differentiation of neurons within the adult VZ, or the initial departure of those neurons already generated, or both.

### Subventricular Neurons within VZ Explants Continued to Express Estrogen Receptor

We next considered the possibility that the decay with time in estrogen's influence on these cultures could have resulted from the loss *in vitro* of estrogen-receptive subventricular neurons in the parent explants. This was of concern since the ability of resident neurons to generate estrogen-dependent paracrine agents may be critical to estrogen's effects in this preparation. However, using whole-mount immunocytochemistry for estrogen-receptor protein (ERP) in adult finch HVC VZ explants fixed after 7–8 or 13–14 days *in vitro*, we observed abundant ERP<sup>+</sup> cells, whose numbers remained stable through the end of the second week in culture (Table 3). These ERP<sup>+</sup> cells were not noticeably different in distribution or number, whether the cultures

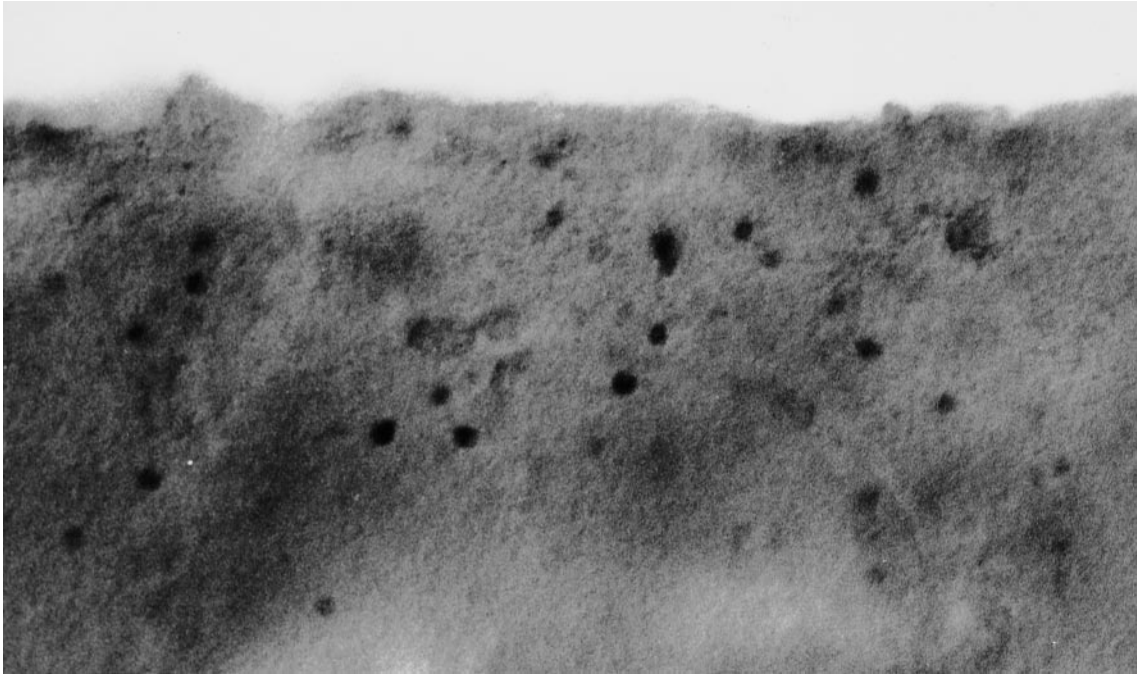
were raised in the presence or absence of added estradiol (Fig. 6). Since no significant differences were noted between 1 and 2 weeks *in vitro* in either the immunoreactivity or number of ERP<sup>+</sup> cells in these explants, estrogen's diminished influence with time in these cultures could not be readily attributed to any decrement in estrogen-receptive target cells, or by inference, in estrogen-dependent paracrine agents. Rather, it was the response of the new migrating neurons to estrogen-associated environmental signals that appeared to be transient and developmentally restricted.

**TABLE 3**

Estrogen Receptive Cells Persisted in Adult Finch HVC Explant Cultures, Beyond the Point at Which Estrogen-Dependent Neuronal Calcium Responses to NgCAM Had Abated

Days <i>in vitro</i> (DIV)	Number of estrogen-receptor immunoreactive cells/explant	
	Estrogen-supplemented media	Estrogen-deficient media
7–8	10 ± 7.9 (n = 7)	11 ± 8.2 (n = 10)
12–13	16 ± 6.2 (n = 6)	10 ± 8.7 (n = 5)

*Note.* Mean ± standard deviation. No significant differences were noted between groups, either by Fisher's exact test or by paired *t* tests adjusted for multiple comparisons.



**FIG. 6.** Explants of the adult finch VZ harbored estrogen receptor-immunoreactive neurons. This photo shows the ventricular edge of an adult female finch neostriatal explant, immunostained for estrogen receptor protein (ERP) after 8 DIV. A total of 42 ERP<sup>+</sup> cells were distributed through serial focal planes of this roughly 300- $\mu$ m-deep, coronally oriented sample. The persistence of these cells may permit estrogen-dependent paracrine agents to continue to be produced *in vitro*. Scale, 50  $\mu$ m.

## DISCUSSION

These data indicate that differentiation and/or departure of new neurons from the adult songbird ventricular zone may be regulated by both estrogenic and androgenic gonadal steroids. Estrogen serves an important additional function in regulating migration from the VZ, in that it is required for the coupling of calcium-dependent signal transduction pathways to neuronal NgCAM, an event that is critically associated with the progression of migration by these cells (Goldman *et al.*, 1996a).

### ***Newly Migratory Adult Neurons Reversibly Couple NgCAM Expression to Calcium Signaling Pathways***

We previously reported that the departure of new neurons from the adult VZ is accompanied by their acquisition of a cellular calcium response to NgCAM recognition, which lasts only for the first several days of migration *in vitro*, even though NgCAM expression by these cells is sustained long thereafter (Goldman *et al.*, 1996a). The time period during which NgCAM elicited its calcium response *in vitro* corresponded to the postmi-

totic age at which newly generated neurons leave the VZ to enter the brain parenchyma *in vivo* (Barami *et al.*, 1995). These results suggested that the calcium response of a newly generated neuron might represent a competence signal, allowing migration to proceed beyond the point of initial departure from the VZ. The present study was intended to identify endogenous regulators of the CAM-dependent calcium response. We found that estrogen, or estrogen-induced factors, are indeed required for the development of NgCAM-dependent calcium signaling and that this process is associated with the extent of neuronal outgrowth from the adult VZ.

### ***Role of NgCAM-Dependent Calcium Signaling during Neuronal Migration***

A threshold degree of NgCAM-dependent calcium activation, occurring only during a restricted period of neuronal ontogeny, might be required for the achievement and maintenance of neuronal response thresholds to environmental signals encountered during initial migration (Goldman *et al.*, 1996a). As such, the transient NgCAM-dependent calcium signaling exhibited by these neurons might parallel the calcium-dependence of neu-

ronal migration noted by Komuro and Rakic (1996). In this regard, any alteration in calcium homeostasis effected by NgCAM activation, whether by NgCAM in the local neuropil, or by heterophilic ligands in the extracellular milieu, might influence the new neuron's threshold to respond to humoral trophins encountered during migration (Koike and Tanaka, 1991). Such a mechanism could be particularly important in modulating the ability of factors such as brain-derived neurotrophic factor (BDNF) to support the postmitotic maturation and survival of neurons arising from the adult SVZ (Kirschenbaum and Goldman, 1995; Goldman, 1997). More broadly, both transient and sustained calcium signals can influence MAP kinase-dependent transcriptional activation (Hill and Treisman, 1995; Lev *et al.*, 1995), introducing a plethora of transcription-dependent means by which calcium transients during initial migration might influence neuronal phenotype and fate.

### **Transduction of the NgCAM-Induced Calcium Signal**

The means by which NgCAM activation results in a cellular calcium response remain controversial. NgCAM itself cannot transmit a calcium signal; it has no intrinsic kinase or phosphatase activity, and no known interactions *in cis* with either voltage- or ligand-gated calcium channels. Instead, NgCAM may bind *in cis* to a heterotypic partner, the activation of which in turn induces a cellular calcium response. Both cytoplasmic tyrosine kinases, *c-src* in particular (Atashi *et al.*, 1992; Ignelzi *et al.*, 1994), and the membrane-linked tyrosine kinase of the FGF receptor (Williams *et al.*, 1994) have been proposed to serve this role, as potential mediators of the NgCAM-stimulated calcium response. In particular, Doherty and Walsh and their colleagues have proposed that the neuronal calcium response to NgCAM/L1 may be initiated through the binding of NgCAM to a CAM-homology domain (CHD) spanning amino acids 151–170 of FGF-R1 (Williams *et al.*, 1994; Hall *et al.*, 1996; Saffell *et al.*, 1997); this segment of FGF-R1 may recognize an homologous portion of the NgCAM/L1 extracellular domain (Hlavin and Lemmon, 1991). The interaction of the two may occur *in cis*, in the plane of the neuronal membrane, which would allow the calcium response to NgCAM to be initiated by the intrinsic tyrosine kinase activity of the NgCAM-activated FGF-R1. As such, the expression or activity of FGFR might be rate-limiting in the establishment of the CAM-dependent calcium signal. Estrogen, either acting itself through receptor-independent means, or through its dependent paracrine effectors, might then serve to

upregulate FGFR-dependent signal transduction, and by so doing permit the new migrant to recognize an NgCAM environment with a rise in somal calcium. These scenarios notwithstanding, the specific means by which estrogen or its induced agents permits the development of NgCAM-dependent calcium signaling, specifically by new neurons entering a parenchymal environment, remains unclear.

### **Estrogen May Act through Paracrine Intermediaries**

It is important to note that new neurons arising from the adult finch VZ do not express estrogen receptor at any time during their migration or maturation (Hidalgo *et al.*, 1995). Instead, the mitotically quiescent estrogen-receptive neurons of the subventricular parenchyma appear to be the primary targets of estrogen in this system. As a result, estrogen is likely to be acting through secondary paracrine intermediaries, that in turn may be targeting either the migrating new neurons or their forebears still within the VZ. In this regard, a principal estrogen-induced paracrine hormone in the uterus is insulin-like growth factor-1 (IGF-1) (Norstedt *et al.*, 1989; Sahlin *et al.*, 1994). IGF-1 has a panoply of cellular effects (de la Rosa *et al.*, 1994; de Pablo and de la Rosa, 1995); on CNS neurons, its two principal described effects are the support of neuronal precursor survival and responsiveness to bFGF (Drago *et al.*, 1991), and the down-regulation of *N-cadherin* (Roark *et al.*, 1992). Each of these effects is potentially important in this system: By preserving bFGF responsiveness and precursor viability, IGF-1 might increase the pool of precursors and their daughter neurons in each VZ explant. Second, since the departure of new neurons from the VZ appears to require the down-regulation of *N-cadherin* (Barami *et al.*, 1994), and since IGF-1 promotes neuronal outgrowth from the adult VZ (Jiang *et al.*, 1998), IGF-1 might serve to induce neuronal departure from the VZ by down-regulating *N-cadherin*, and may do so in an estrogen-dependent fashion. Together, these observations suggest a means by which estrogen, working through a paracrine intermediary, might promote neuronal departure from the VZ, and hence neuronal outgrowth *in vitro*.

### **Estrogen Contributes to the Outgrowth of New Neurons from the Adult Ventricular Zone in Vitro**

We found that estrogen supported the initial appearance of neurons from explants of the adult VZ: During the first 8 DIV, those explants exposed to estrogen exhibited consistently higher numbers of neurons in

their cellular outgrowths than their counterparts raised in steroid-depleted media. This finding may correspond to our prior observation that both the number and proportion of [<sup>3</sup>H]thymidine-labeled neurons that survive the first postmitotic month are substantially higher in estrogen-treated castrated canaries than in their untreated counterparts (Hidalgo *et al.*, 1995). Since estrogen does not appear to influence the mitotic rate of VZ precursor cells, its trophic effects, presumably exercised through paracrine intermediaries, must act upon newly post-mitotic neurons, rather than their dividing progenitors (Brown *et al.*, 1993; Burek *et al.*, 1995; Hidalgo *et al.*, 1995).

Estrogen's effect on new neurons may be explained by either their increased survival or by an estrogenic facilitation of neuronal migration from the VZ. In fact, no long-term effects of either estrogen or testosterone were noted in these cultures; in both finches and canaries, the survival curves were roughly parallel in the gonadal steroid-supplemented and control plates (Fig. 5). Although both testosterone and estradiol displaced the survival curves of both species to the right, the greater initial abundance of neurons in the steroid-treated groups appeared to account for most of this difference. Far more striking were the estradiol- and testosterone-associated elevations of the growth curve. These effects suggested that the gonadal steroids may serve to promote either neuronal differentiation or initial ventricular zone departure, or both. Yet neither estradiol nor testosterone acted directly to confer any long-term survival advantage to adult VZ-derived neurons.

### **Estrogen Targets Remained Viable in These Cultures**

Certainly, neuronal survival might be serially dependent upon other neurotrophic agents, after a transient period of estrogen-dependent outgrowth and/or survival. However, a number of methodological considerations might also explain the brief time course of the estrogen effect: The size of each explant and the number and density of ER<sup>+</sup> neurons that it contains, as well as the long-term survival of these ER<sup>+</sup> cells within the explant, might influence the ability of added estradiol to exert paracrine effects upon new neurons. We therefore considered the specific possibility that estrogen-responsive subventricular cells might have a limited *in vitro* survival and thereby be rate-limiting for estrogen-dependent neuronal outgrowth and calcium-signaling to NgCAM. We found that the number of immunodetectable estrogen-receptor expressing neurons in each ex-

plant was in fact stable between 7 and 13 days in culture (Fig. 6; Table 2). As noted previously, calcium signaling to NgCAM by newly generated neurons is transient, extending no later than 9 DIV (Goldman *et al.*, 1996a). Thus, estrogen-receptive cells persisted in these cultures long after the point at which the calcium responses to NgCAM of newly migratory neurons were lost. As a result, the marked decline in calcium response to NgCAM with maturation did not seem attributable to any artifactual loss of estrogen-receptive cells in these cultures. Similarly, the difference in neuronal outgrowth between estrogen-supplemented and unsupplemented plates could not be readily explained on the basis of any temporal decay in the number of estrogen-receptive neurons; to the contrary, estrogen-receptive cells persisted in these cultures beyond the point at which the new neurons themselves had largely died.

### **Testosterone Did Not Trigger the Development of NgCAM-Mediated Calcium Signaling**

The coupling of NgCAM to its dependent calcium signaling pathways was elicited by exposure to estrogen, but not to androgen (Table 1; Fig. 3): The testosterone-treated adult VZ cultures exhibited no more calcium response to NgCAM than did their steroid-deficient controls. The restriction of signal competence to estrogen-treated neurons was particularly striking in light of the similar promotion of neuronal outgrowth offered by both estrogen and testosterone: In both finches and canaries, testosterone, like estrogen, promoted the initial differentiation and/or departure of new neurons from these HVC explants *in vitro* (Fig. 5). Together, these findings suggest that estrogen's effects on neuronal recruitment from the VZ, shared by testosterone, are distinct from its subsequent induction of NgCAM-dependent calcium signaling. Paradoxically, systemic testosterone may be the rate-limiting determinant of each of these processes *in vivo*, since estradiol is generated locally within the neostriatal parenchyma by the action of brain aromatase upon serum testosterone (Schlinger and Arnold, 1991; Shen *et al.*, 1995).

In this regard, it is important to note that operational limitations of the explant culture system may have prevented the development of CAM-calcium coupling to testosterone: Although aromatase is produced *in vitro*, and continues to convert testosterone to estradiol, net aromatase activity is a function of glial cell number (Schlinger *et al.*, 1994). Yet astrocytic and ependymal outgrowth and expansion only begin after 4 DIV in these cultures, so that aromatase activity in the first week may be restricted to that of the parent explant. The

glial outgrowth observed in the first few days *in vitro* may therefore be insufficient to produce enough aromatase to achieve the threshold amount of estradiol required for acquisition of calcium signaling to NgCAM. By the time significant glial expansion has occurred, the 6–8 DIV window during which NgCAM-triggered calcium signaling normally occurs might already have closed.

### Overview

We have found that both the departure of new neurons from explants of the adult songbird VZ, and the coupling of new neuronal NgCAM to its dependent calcium signaling pathways, are strongly promoted by estrogen treatment. These effects appear to be sequential and dissociable, in that testosterone similarly supports neuronal outgrowth from the VZ, but does not elicit the development of NgCAM-dependent calcium responses. Together, these findings indicate that the initial recruitment of new neurons from the adult ventricular zone can be modulated by gonadal hormones. In addition, these observations suggest a novel means by which gonadal hormones and their paracrine intermediaries might modulate brain ontogeny, by dynamically regulating the coupling of cell adhesion molecules to their dependent calcium signaling cascades during initial migration.

## EXPERIMENTAL METHODS

### Adult Explant Cultures

**Culture preparation.** Both zebra finches (*Poephilia guttata*) and canaries (*Serinus canarius*, American Singer str.) were used in these experiments. Both were used in the analyses of neuronal outgrowth and viability as a function of hormone treatment, whereas only finches were used for assessing the effects of NgCAM on neuronal calcium. Cultures were prepared from the neostriatal VZ, both overlying and medial to nucleus HVC, as described (Goldman, 1990; Goldman *et al.*, 1992).

**Media base.** Our culture medium was based upon previously reported formulations (Bottenstein and Sato, 1979; Goldman *et al.*, 1992): To phenol red-free DMEM/F12 with 15 mM HEPES, we added glutamine (2 mM), glucose (6 mg/ml), insulin (6 µg/ml), transferrin (6 µg/ml), selenium (6 ng/ml), linoleic acid-rich (5 µg/ml) albumin (1.25 mg/ml), hydrocortisone (300 ng/ml);

water-soluble preparation, Sigma H0396), progesterone (60 fg/ml), putrescine (16 µg/ml), and tri-iodothyronine (30 ng/ml). To this base, we added charcoal-stripped fetal bovine serum (Hyclone) and castrated rooster (capon) serum (Cocalico), each at 10% v/v. The charcoal-stripped serum was assayed to contain 330 pg/ml T3 and 1.3 uU/ml insulin, each representing an approximate 80% reduction in their initial concentration (assayed by Hyclone Labs). Estradiol, testosterone, and androstenedione were all undetectable in both the stripped sera and resultant depleted medium (Mayo Clinic Labs; see Hidalgo *et al.*, 1995, for assay description).

**Gonadal steroid supplements.** To the base medium was added one of several possible gonadal steroid supplements, prepared from water-soluble cyclodextran-conjugated preparations. These included estradiol (1 ng/ml; Sigma E4389), testosterone (10 ng/ml; Sigma T5035), or cholesterol (10 ng/ml, Sigma C4951).

### Assessment of *in Vitro* Neuronal Survival

**Cell counts.** For neuronal outgrowth and survival studies, explants were obtained from either adult female zebra finches ( $n = 4$  birds) or 1 year-old female canaries ( $n = 9$ ). Cultures were prepared from the neostriatal VZ, both overlying and directly medial to nucleus HVC, as described (Goldman, 1990), using the media formulation and treatment additions described above. Once an explant displayed cellular outgrowth, its neurons were identified using morphological criteria that we have established and verified immunocytochemically, ultrastructurally and functionally in this system (Barami *et al.*, 1995; Goldman, 1990; Goldman and Nedergaard, 1992). Neurons within each explant outgrowth were counted every other day beginning on day 5; during this time, hormone supplements were added to the culture media every other day, and one-third volume media changes were performed twice weekly.

**Statistical analysis.** For each explant, the mean and median number of neurons per explant in each treatment group, as well as the standard deviation and error, were calculated. Explant growth patterns were then analyzed by general linear modeling using 2-factor, repeated measures analysis of variance (ANOVA). The time-points (days *in vitro*) at which counts were made constituted the repeated measures factor, whereas the type of steroid treatment (estradiol vs cholesterol) was the main effect of the ANOVA. For statistically significant ANOVA results of  $P < 0.05$ , post hoc pairwise comparisons were made between treatments, using a Bonferroni adjustment for multiple comparisons. Statis-

tical significance of these comparisons was verified by the  $\chi^2$  test using contingency tables.

### Immunocytochemistry

**Estrogen receptor.** Immunolocalization of estrogen receptor protein (ERP)-immunoreactivity was accomplished using a rat monoclonal antibody directed against estrogen receptor protein (mAb H222SP $\gamma$  IgG, Abbott Labs). This antibody recognizes both the unoccupied estrogen receptor and the ligand bound hormone receptor complex (Greene *et al.*, 1984; Miller *et al.*, 1982) and effectively localizes estrogen receptor protein in Passerine songbirds (Gahr, 1990; Hidalgo *et al.*, 1995). Cultures of 7 and 13 DIV zebra finch HVC were placed in 0.1% Triton X-100 for 30 min, washed in 0.1 M phosphate buffer (PB), exposed to 10% normal rabbit serum for 30 min, then monoclonal antibody H222SP $\gamma$ , a rat anti-estrogen receptor protein IgG, at 1:1000 overnight at 4°C. After washing, the cultures were incubated in biotinylated rabbit anti-rat IgG (1:200; Vector Laboratories) at 25°C for 90 min, rewashed, incubated in avidin-biotin-HRP complex (Vectastain ABC; Vector) for 60 min, washed again, then developed with diaminobenzidine (0.2 mg/ml) and 0.003% hydrogen peroxide for 5 min. The explants were then washed, cleared through ascending alcohols and xylene, mounted in Permount, and photographed using a Leitz Laborlux photomicroscope. Control cultures were treated similarly except for the omission of the primary or secondary antibodies; no cells were labeled in these sections.

**NgCAM.** Immunostaining for NgCAM was done using rabbit anti-zebra finch NgCAM (Barami *et al.*, 1994). Cultures were fixed for 5 min in cold 4% paraformaldehyde, washed, and serially exposed to 0.1% saponin/1% NGS for 15 min, 0.05% saponin/5% NGS for 15 min, then ammonium sulfate-precipitated anti-finch NgCAM Ig (1:100) for  $\geq 2$  h, all at room temperature. Cultures were then washed with PB and exposed to biotinylated goat-anti rabbit IgG (1:50) for 30 min. Cultures were washed again in PB and then exposed to fluorescein-conjugated avidin (Vector), 1:25 in PB (pH 8) for 30 min, washed in PB, then mounted in SlowFade, and examined under fluorescence microscopy.

### Assessment of the Effects of NgCAM on Neuronal Calcium

**Antigen challenge.** The confocal-assisted calcium imaging data reported here derived from a total of 26 explant outgrowths, sampled from 6 adult female zebra

finches. Prior to NgCAM addition, each finch VZ explant was exposed to a vehicle control (DMEM). A stable calcium baseline among cells within the selected field was then assured for at least 15 min, before the test antigen was added. Matched controls were also run, that received vehicle in place of NgCAM. Avian NgCAM protein was immunopurified from chick brain using monoclonal antibody 8D9 (Lemmon and McLoon, 1986). In the experimental plates, NgCAM (1.2  $\mu\text{g}/\text{ml}$ ) was added after 7 DIV.

**Calcium measurement using confocal imaging of fluo-3.** Each culture was loaded with 10  $\mu\text{M}$  fluo-3 acetomethoxyester (fluo-3 AM, Molecular Probes) (Minta *et al.*, 1989) for 1 h at 37°C. A Bio-Rad MRC600 confocal scanning microscope, coupled to an Olympus IMT-2 inverted microscope, was used to image the fluo-3 signal. Excitation was provided by the 488 nm line of a 25 mW argon laser, neutral density-filtered to 0.1%. Emission was long pass-filtered (515 nm) and detected with the confocal set to its maximal aperture (7 mm). Images were acquired every 5 min using Comos 7.0 (Bio-Rad) and recorded on a Panasonic TQ-2028F optical disc recorder. At the completion of each experiment, cultures were challenged with 60 mM K<sup>+</sup>, to assess the integrity of their calcium responses to depolarization. Relative changes in fluorescence were then calculated and normalized against baseline fluorescence by  $\Delta F/F$  (Connor *et al.*, 1987; Kirschenbaum *et al.*, 1994; Nedergaard, 1994). Background counts were subtracted from all experiments, each of which was carried out at 25°C in HBSS.

**Calcium measurement by ratio imaging with fura-2.** Selected cultures were loaded with 2  $\mu\text{M}$  fura-2 AM in their culture media at 37°C. Sixty minutes later, the culture-bearing coverslips were washed in fresh media and mounted in a Leiden incubation chamber on an inverted microscope (Olympus IX70). The microscope was equipped with xenon/mercury bulb, a filter wheel (lambda-10, Sutter Instrument Corp.) controlled by Universal Imaging software, band pass filters (340  $\pm$  5; 380  $\pm$  5), and an emission filter 510 (ChromaTech). The cultures were viewed with a  $\times 20$  0.75 N.A. fluor objective. Ca<sup>2+</sup><sub>i</sub> was measured every 30 s. Measurement of the fluorescence signal in terms of free Ca<sup>2+</sup><sub>i</sub> was based upon the procedure described by Grynkiewicz *et al.*  $R_{\text{min}}$  and  $R_{\text{max}}$  were 0.136 and 2.9 in our system, and a  $K_d = 225 \times 10^{-9}$  M was used. For each measurement, the background signal was determined as the emission signal, in a representative field of the same size in an unloaded sister culture; this background level was then subtracted from each experimental measurement.

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