

Transient Coupling of Ng-CAM Expression to NgCAM-Dependent Calcium Signaling during Migration of New Neurons in the Adult Songbird Brain

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The adult avian forebrain continues to generate neurons from subependymal zone (SZ) precursor cells, whose neuronal progeny migrate into the brain upon radial guide fibers. These neurons express the immunoglobulin-family adhesion molecule NgCAM, and their migration in culture is disrupted by anti-NgCAM Fab. Confocal imaging of adult zebra finch SZ loaded with the calcium indicator fluo-3, as well as ratio imaging with the indicator fura-2, revealed that migrating new neurons responded to microgram amounts of NgCAM with reversible increments in cytosolic calcium. The calcium response to NgCAM antigen was developmentally restricted, in that it was only manifested by neurons for roughly the 3- to 4-day period between 6 and 9 DIV, even though NgCAM expression persisted tonically thereafter. The period during which NgCAM elicited a calcium signal corresponded to the postmitotic age at which new, bipolar neurons leave the adult SZ to enter the brain parenchyma *in vivo*. Accordingly, the calcium response to NgCAM was largely limited to morphologically bipolar cells. Anti-NgCAM IgG also evoked a neuronal calcium signal over the same restricted period that NgCAM protein exerted its effect. These findings suggest a dynamic coupling and uncoupling of calcium-dependent signal transduction pathways to a stably expressed surface adhesion molecule, whose function in a given neuron may therefore evolve with cellular maturation.

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INTRODUCTION

The adult avian forebrain continues to generate neurons from subependymal zone (SZ) precursor cells (Goldman and Nottebohm, 1983), whose neuronal progeny then migrate into the brain along radial guide fibers (Alvarez-Buylla and Nottebohm, 1988). These radial guide fibers are ependymal in phenotype and coderive with new neurons from a common precursor (Goldman *et al.*, 1993b). The early postmitotic neuronal specification of these cells is accompanied by their expression, while still in the SZ, of neuronal RNA-binding proteins of the Hu family (Barami *et al.*, 1995). Hu immunoreactive, neuronally specified cells persist within the adult SZ for at least 4 days after their parental precursor cell division and only leave the SZ after down-regulating the surface adhesion molecule *N*-cadherin, which is tonically expressed by cells of the SZ, including the neuronal precursors themselves (Barami *et al.*, 1994). Upon down-regulating *N*-cadherin and departing the SZ for the subjacent parenchyma, the new neurons express the immunoglobulin superfamily adhesion molecule NgCAM, whose expression is maintained throughout neuronal migration and beyond. In explant cultures of the adult finch SZ, exposure of the new neurons to anti-NgCAM IgG disrupted their migration upon ependymally derived radial guide cells and impaired their subsequent viability (Barami *et al.*, 1994).

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 (Lindner *et al.*, 1983, 1986; Grumet *et al.*, 1983). However, the late death of migrating adult SZ-derived neurons exposed to anti-NgCAM suggested that the role of NgCAM activation in this system might extend beyond intercellular adhesion. This possibility was particularly intriguing in light of recent findings that (1) the binding of both L1 and anti-L1/NgCAM to cognate neuronal receptors is associated in some neurons with a G-protein-mediated influx of calcium through voltage-dependent calcium channels (Schuch *et al.*, 1989; von Bohlen *et al.*, 1992; Asou, 1992), (2) anti-L1/NgCAM-associated calcium increments are associated with an inhibition of pp60^{c-src} dependent tyrosyl phosphorylation of tubulin (Atashi *et al.*, 1992), and (3) neurite outgrowth supported by L1 can be inhibited by calcium channel antagonists (Williams *et al.*, 1992). These findings suggested that NgCAM activation might represent the initial step in a calcium-dependent signaling cascade leading to functional neurotrophism, over and above the calcium-independent adhesive interactions subserved by NgCAM binding to its receptor.

In the present study, we used laser-activated confocal imaging of cultures of the adult zebra finch SZ, loaded with the calcium indicator dye fluo-3, to examine the responses of new neurons to added NgCAM protein. We found that migrating new neurons responded to microgram amounts of NgCAM with reversible increments in cytosolic calcium. Furthermore, the neurons responded to NgCAM with a calcium increment only during a discrete period of development, corresponding to their initial parenchymal migration. The neurons exhibited a similar calcium response to anti-NgCAM IgG, and did so over the same restricted time course. However, neurons continued to express NgCAM immunoreactivity for at least a week after they ceased to respond to its addition with a calcium signal. Thus, the capability of newly generated neurons to transduce an NgCAM/L1-mediated calcium signal may be regulated independently of the cells' expression of NgCAM/L1 protein.

RESULTS

New Neurons Responded to NgCAM with Reversible Increments in Cytosolic Calcium

Explant cultures of the neostriatal subependymal zone were prepared from adult zebra finches (Goldman, 1990; Goldman *et al.*, 1992; Goldman and Nedergaard, 1992). By 5 DIV, productive explants generated an out-

growth of initially bipolar postmitotic neurons, with maturation to multipolarity evolving over the 2–6 days thereafter. When 7 DIV cultures were loaded with the calcium-sensitive dye fluo-3 and examined by confocal microscopy during exposure to immunopurified chicken NgCAM (1.2 $\mu\text{g}/\text{ml}$), newly generated and migratory adult neurons displayed increments in cytosolic calcium that were appreciable within 5 min after NgCAM addition (median of 7 experiments, including 192 neurons) (Fig. 1). These increments were sustained, rising to an average maximum Ca_i^{2+} of $130 \pm 22\%$ over baseline, by 52 ± 9 min after the onset of continuous NgCAM exposure (Table 1).

The Calcium Response to NgCAM Was Developmentally Restricted

Whereas most neurons responded to NgCAM with a calcium signal by 7 DIV, only a minority were competent to do so at 5 DIV, and these demonstrated average calcium increments of only $39 \pm 17\%$ ($n = 85$ scored neurons). The calcium responses to NgCAM attenuated with subsequent neuronal maturation, so that by 11 DIV only a statistically insignificant Ca_i^{2+} rise of $14 \pm 15\%$ ($n = 125$ cells) was noted in response to NgCAM (Figs. 2 and 3).

Not only were the per cell calcium responses to NgCAM highest among neurons in the 7-DIV group, but the average proportion of neurons responding to NgCAM was also maximal at that time. Thus, while less than half of the neurons responded to NgCAM with a $>20\%$ calcium increment at 5 DIV (46% of 56 neurons scored), most did so after 7 DIV (77%, or 162 of 192 neurons). In contrast, by 11 DIV only 28% (29/125) responded so (Table 1).

Migrating Bipolar Neurons Were Those Responsive to NgCAM

The age-dependent change in the proportion of neurons responding to NgCAM reflected morphological maturation, in that while most migrating, bipolar and fusiform neurons responded to NgCAM with a calcium increment, relatively few multipolar neurons did so, and those to a significantly lesser degree (Fig. 4). Among 99 neurons categorized morphologically and exposed to 1.2 μg NgCAM at 7 DIV, bipolar neurons ($n = 62$) displayed a mean calcium increment of $292 \pm 27\%$, in contrast to the $82 \pm 13\%$ rise exhibited by tripolar neurons in the same outgrowths ($n = 37$; $P < 0.01$).

TABLE 1

Effect of NgCAM Protein upon Ca_i^{2+} in New, Adult SZ-Derived Neurons

Condition	DIV	Cells counted ^a	% Neurons with Ca rise >20%	Average maximum Ca rise (%) ^b
As a function of the number of days of neuronal maturation <i>in vitro</i>				
NgCAM, 1.2 μg^c	5	85	48	39 \pm 17*
NgCAM	7	192	77	130 \pm 22*
NgCAM	11	125	28	14 \pm 15
With perturbation of calcium homeostasis				
NgCAM + Ca-free media ^d	7	50	12	-19 \pm 7**
NgCAM + nifedipine, ^e 10 μM	7	41	24	0 \pm 9**
NgCAM + staurosporin, ^f 10 nM	7	60	43	30 \pm 14**
NgCAM + pertussis, ^g 0.5 $\mu\text{g}/\text{ml}$	7	66	42	10 \pm 14**
NgCAM + ω -conotoxin, ^h 1 μM	7	89	54	59 \pm 19***
NgCAM + thapsigargin, ⁱ 2 μM	7	162	59	56 \pm 13***
Controls				
Control: vehicle	7	62	27	6 \pm 9
Control: N-cadherin, 2 μg	7	58	36	17 \pm 9

^a At least 40 cells were scored in each treatment group. These were sampled from an average of 3 cultures/treatment (range: 2–7 plates/group). All values stated as means \pm standard error of the mean (\pm SE).

^b This value represents the average maximal Ca increment of all neurons tested in each group, not just the responders.

^c All NgCAM additions were adjusted to 1.2 $\mu\text{g}/\text{ml}$.

^d Calcium-free HBSS supplemented with 3 mM Mg, 2 mM EGTA.

^e Nifedipine (Sigma), ^fstaurosporin (Gibco), ^h ω -conotoxin (Gibco) and ⁱthapsigargin (Sigma) were all added 10–20 min before NgCAM addition, while ^gpertussis toxin (Sigma) was added 1–14 h before NgCAM. In all cases, NgCAM was added 15 min after confirmation of a stable Ca baseline.

* Significant increase in Ca_i^{2+} relative to vehicle control, to $P < 0.05$.

** Significant inhibition in Ca_i^{2+} rise relative to NgCAM alone, at 7 DIV; also $P < 0.05$. Comparisons between drug treatments by *t* test with Bonferroni adjustment for multiple tests. Comparison of 5-, 7-, and 11-DIV NgCAM and control additions also done by one-way ANOVA with post hoc *t* tests, also with Bonferroni adjustment.

Neurons Expressed NgCAM Long after Their Calcium Responses to It Abated

The neuronal response to NgCAM was uncoupled from NgCAM expression during progressive maturation: Over the period during which NgCAM-dependent calcium responses evolved and abated, the expression of NgCAM did not vary substantially among neurons in these cultures, either as a function of postmitotic age or morphology. NgCAM-immunoreactivity was readily demonstrated on the neurons upon their departure from the explants and persisted for at least 14 DIV. Under equivalent staining and culture conditions, single-cell immunostaining for NgCAM did not noticeably vary over this period (Fig. 5). Thus, neurons expressed NgCAM long after their calcium responses to it had abated. In addition, the ability of these cells to conduct

a voltage-dependent calcium current remained robust, even after their period of calcium response to NgCAM had passed; at 11 DIV, 105 of 109 neurons responded to depolarization with 60 mM KCl (98%), and these with a mean calcium increase of $531 \pm 56\%$.

Neurons Exhibited an Analogous Calcium Response to Anti-NgCAM IgG and with the Same Time Course of Signal Competence

In previous experiments, we had observed that antibodies against NgCAM disrupted the *in vitro* migration of new neurons from the adult finch SZ and impaired the subsequent survival of neurons so affected. The diminished survival of neurons exposed to anti-NgCAM appeared to be specific to the anti-NgCAM/neuronal in-

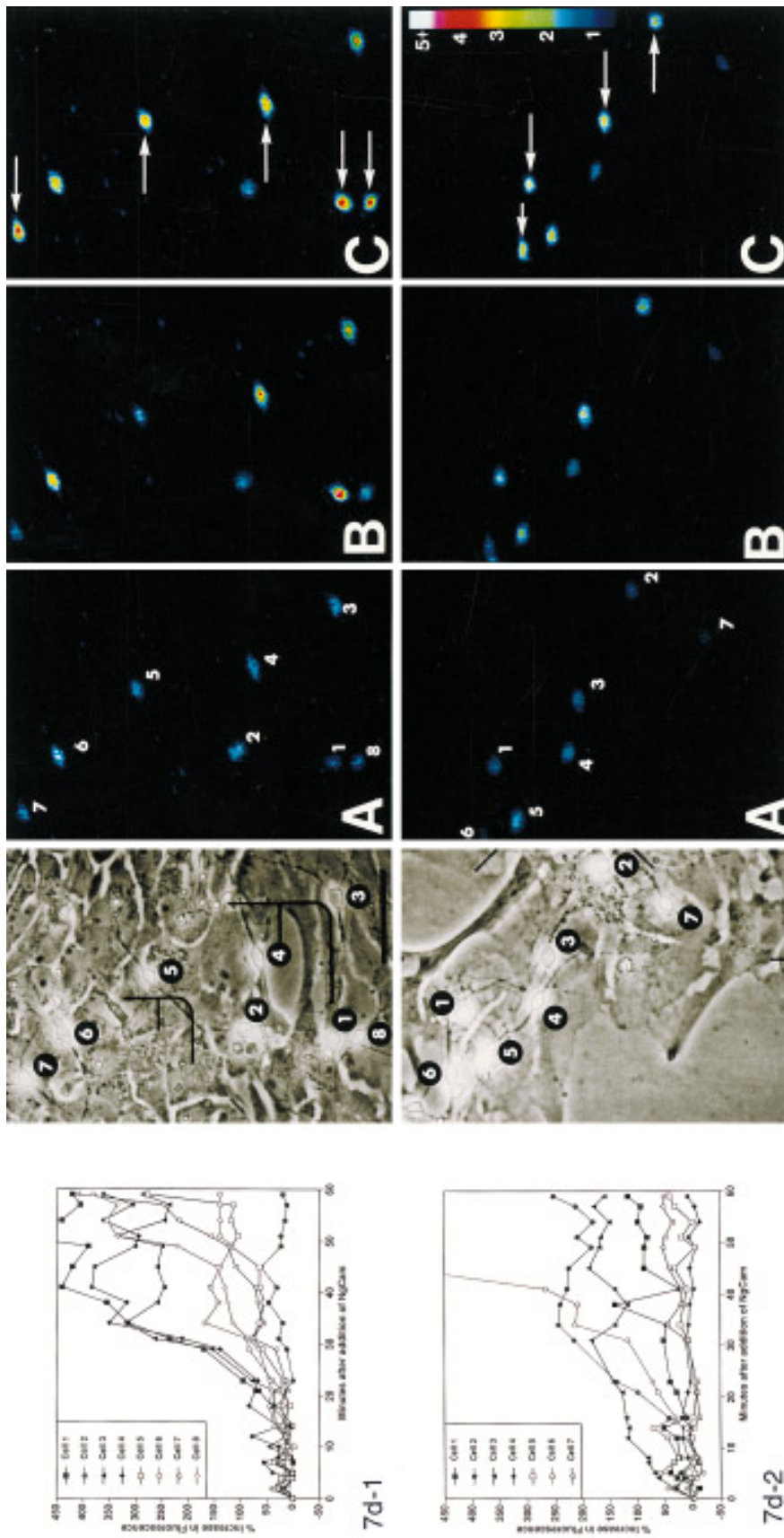


FIG. 1. NgCAM protein elicits a calcium response by new adult SZ-derived neurons. These figures show representative examples of neuronal fields exposed to NgCAM at 7 DIV. Among those neurons that responded to NgCAM, significant calcium increments were noted within 5 min after addition, even though maximal responses were not achieved until 52 ± 9 min after challenge. In this figure, each row should be read left to right: In each, the left graphs the Ca^{2+} levels of each neuron in the selected field, as a function of time. Each neuron can be followed from plate to plate by its numerical designation, as noted on each adjacent phase micrograph. For each time point, A represents the calcium image of each field just before NgCAM addition, obtained at least 1 h after loading with fluo-3 and 15 min after achieving a stable baseline. B shows each field's calcium response to NgCAM addition, while C shows each field's approximate point of maximal response, imaged at 50 min. Among the 15 neurons identified in these two fields, 13 responded to NgCAM with a $>20\%$ increment in the fluo-3 calcium signal, while none of their matched control cultures did so (Table 1). Among the responders (arrows in C), the mean calcium response to NgCAM was $+248 \pm 38\%$.

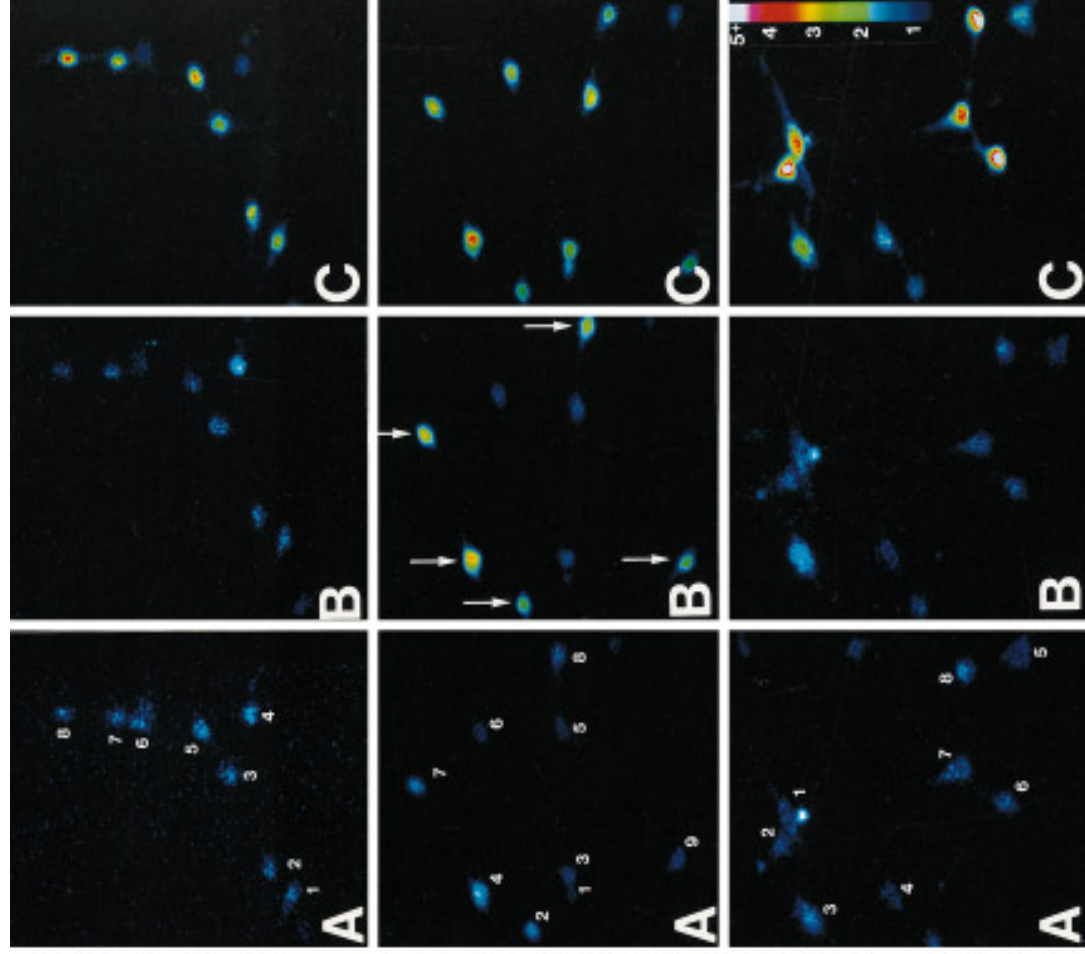
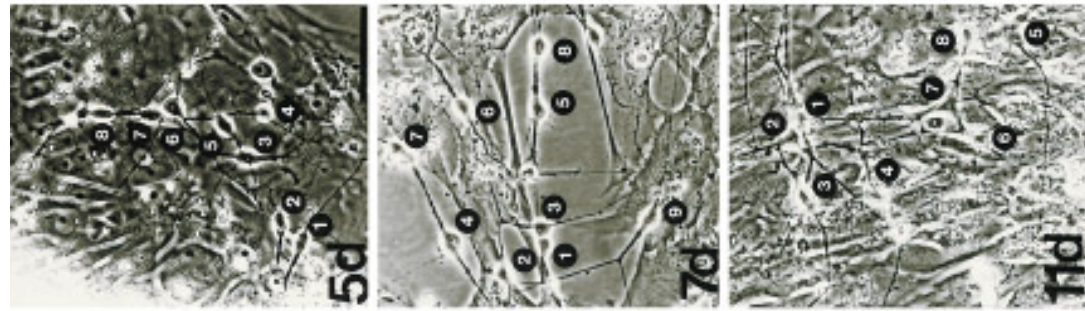
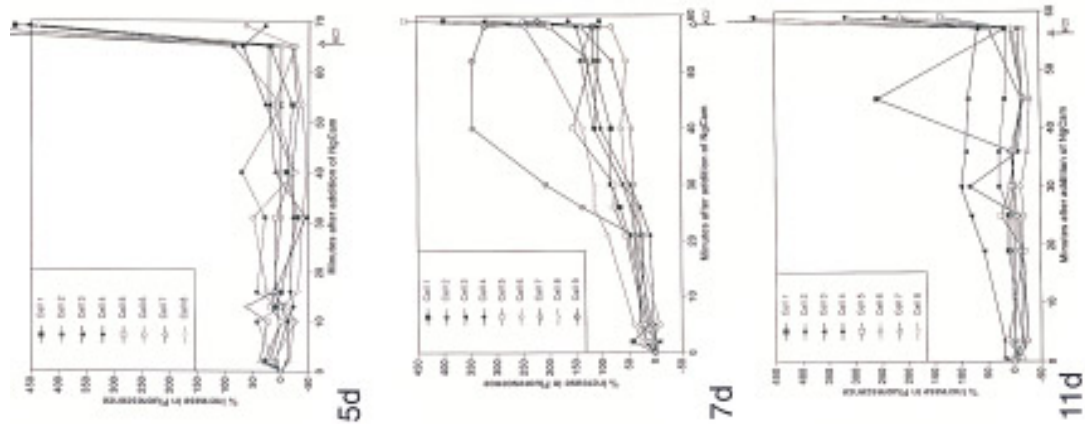


FIG. 2. The neuronal calcium response to NgCAM was temporally restricted. These three rows compare the calcium responses of adult finch SZ-derived neurons at 5, 7, and 11 DIV to 1.2 $\mu\text{g}/\text{ml}$ NgCAM addition. In this figure, the left graphs the Ca^{2+} levels of each neuron in the adjacent 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, 2 h after NgCAM exposure. Whereas neurons in the 5- and 11-DIV cultures show little Ca rise to NgCAM, most of the neurons in the 7-DIV culture respond significantly (arrows). (C) To demonstrate that 5- and 11-DIV neurons, like their 7-DIV counterparts, have voltage-gated calcium channels, 60 mM KCl was added to each culture 2 h after NgCAM addition. At all three time points, neurons responded with rapid influxes of calcium; in the 7-DIV cultures, neuronal Ca increments to KCl depolarization were over fourfold those evoked by NgCAM.

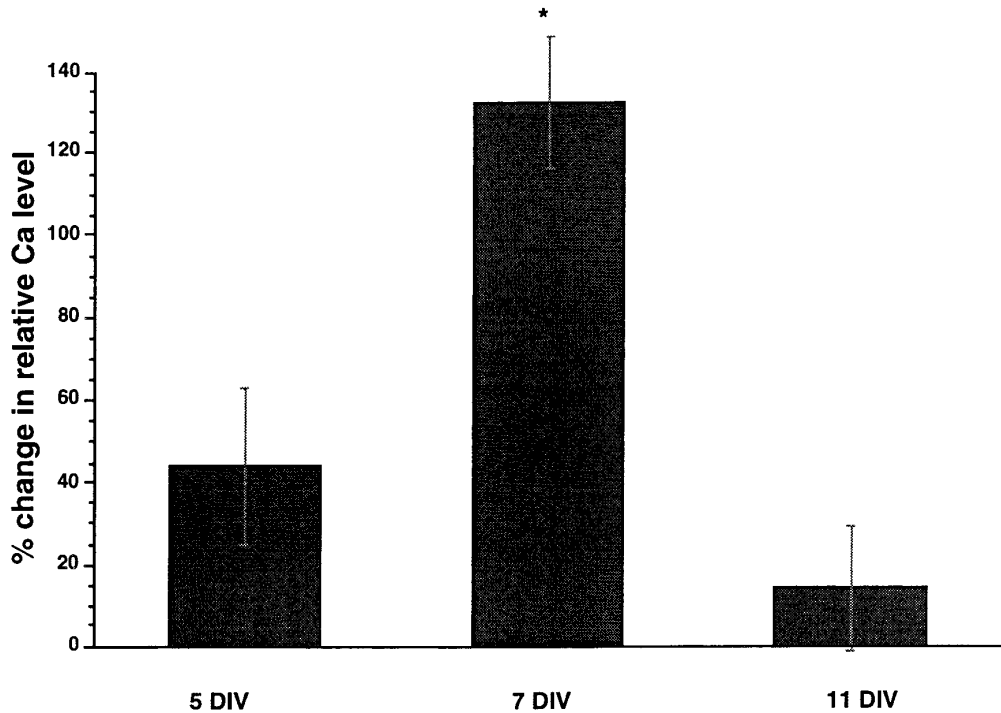


FIG. 3. The calcium response to NgCAM was developmentally restricted. This graph compares the average percentage rise in cytosolic calcium after addition of NgCAM (1.2 $\mu\text{g}/\text{plate}$, or 2 $\mu\text{g}/\text{ml}$) at 5, 7, and 11 DIV. Most neurons responded to NgCAM antigen addition with a rise in cytosolic calcium by 7 DIV, after displaying minimal responses earlier in their postmitotic ontogeny (5 DIV). However, these calcium responses attenuated with maturation and abated by 11 DIV. *Denotes significant difference at $P < 0.01$ relative to both 5- and 11-DIV groups.

teraction. Perturbed survival was not associated with a variety of antibodies against other surface adhesion molecules, including both anti-N-CAM and anti-N-cadherin, or with an assortment of non-cross-reactive anti-NgCAM and anti-L1 antibodies and their vehicles (Barami *et al.*, 1994). Neurons exposed to anti-NgCAM might have suffered as a result of a loss of target-derived support, resulting from neuritic retraction induced by anti-NgCAM exposure. Alternatively, the *trans* activation of surface NgCAM by anti-NgCAM in the extracellular milieu might have yielded a lethal degree and/or duration of calcium entry into the affected neuron, killing it directly. Finally, the blockade of NgCAM reception by anti-NgCAM might have deprived migrating neurons of an NgCAM-dependent signal necessary for their subsequent survival.

To distinguish among these possibilities, we next asked whether anti-NgCAM IgG elicited calcium responses analogous to those elicited by NgCAM protein. To this end, anti-NgCAM IgG (100 $\mu\text{g}/\text{ml}$) was added to adult finch SZ cultures after either 5, 7, 9, or 11 DIV. Neuronal calcium responses to anti-NgCAM IgG were compared among these different time points, as well as to the responses of control neurons exposed to preim-

mune IgG or anti-N-cadherin IgG. We found that the neurons exhibited calcium responses to anti-NgCAM that indeed paralleled their responses to NgCAM protein, with the same temporally restricted period of signal competence (Fig. 6). Neurons responded to anti-NgCAM IgG maximally at 7–9 DIV, after exhibiting little response at 5 DIV, but their calcium responses to anti-NgCAM fell rapidly thereafter and were not apparent at 11 DIV (Table 2). Thus, the time period of postmitotic neuronal development during which neurons exhibited a calcium response to anti-NgCAM antibody corresponded to that during which NgCAM protein elicited its calcium response. This suggested that the anti-NgCAM-induced calcium response also utilized the NgCAM-dependent signaling pathway.

The Calcium Response to NgCAM Protein Was G-Protein Dependent

We next sought to identify the proximal steps in this NgCAM-dependent signaling cascade. In embryonic forebrain neurons, L1-triggered calcium currents are G-protein-dependent and are carried by both L- and N-type calcium channels (Walsh and Doherty, 1992; Williams *et*

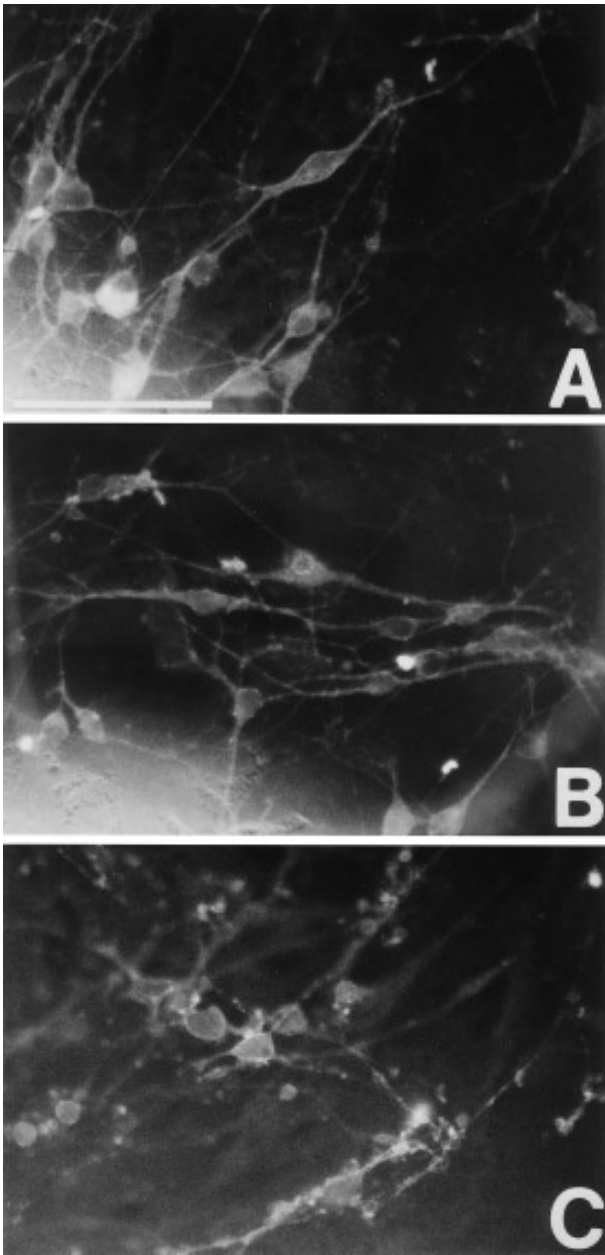


FIG. 5. NgCAM was expressed tonically and persistently by adult SZ-derived neurons, long after their period of NgCAM-linked calcium signaling had abated. Cellular immunostaining for NgCAM was apparent by 5 DIV (A), upon neuronal departure from the SZ explants, and persisted at 7 DIV (B) and 11 DIV (C). Cellular NgCAM immunoreactivity did not appear to vary over this time span, even though the neuronal calcium signal to NgCAM recognition waxed and waned dramatically over this same time span.

al., 1992). We confirmed this to be the case for adult SZ-derived neurons as well: The NgCAM-triggered calcium currents of adult SZ-derived neurons were G-protein-

dependent and carried by both L- and N-type calcium channels. In 7 DIV cultures of the adult finch SZ, the G-protein inhibitor pertussis toxin (PTX) completely inhibited the neuronal calcium response to NgCAM antigen: Whereas 7 DIV neurons exposed to NgCAM (1.2 $\mu\text{g}/\text{ml}$) displayed a $130 \pm 22\%$ maximal increment in Ca_i^{2+} ($n = 192$), their PTX-exposed counterparts exhibited only a $10 \pm 14\%$ rise in Ca_i^{2+} ($n = 66$ neurons), a level that was not statistically different from controls unexposed to NgCAM (Table 1). The Ca_i^{2+} response to NgCAM was composed of extracellular calcium entering the cell, in that exposure of these neurons to NgCAM in calcium-deficient media failed to yield any rise in Ca_i^{2+} . In contrast, treatment with thapsigargin, an inhibitor of endoplasmic reticular Ca^{2+} ATPase that deters the release of calcium from intracellular stores, was associated with only a partial inhibition of the Ca_i^{2+} rise to NgCAM, to $56 \pm 13\%$ ($n = 162$). Furthermore, staurosporin inhibition of cellular protein kinases reduced the NgCAM-induced calcium increment to $30 \pm 14\%$ ($n = 60$), a level not significantly different from controls not exposed to NgCAM (Table 1). This inhibition of NgCAM-dependent calcium entry may have resulted from an inhibition by staurosporin of the protein kinase C (PKC)-dependent phosphorylation of voltage-gated calcium channels. However, staurosporin's dose-dependent inhibition of a variety of other tyrosine kinases leaves moot the specific role of PKC in NgCAM-dependent calcium signaling.

The Calcium Response to NgCAM Was Mediated by L- and N-Type Calcium Channels

Both L- and N-type calcium channels contributed to calcium entry, in that the L-channel blocker nifedipine and the N-channel inhibitor ω -conotoxin each attenuated neuronal Ca_i^{2+} responses to NgCAM: Nifedipine completely abolished NgCAM-activated calcium entry, with a calcium change of $0 \pm 9\%$ to NgCAM ($n = 41$), while in the presence of ω -conotoxin, NgCAM elicited a submaximal rise of $59 \pm 19\%$ in Ca_i^{2+} ($n = 89$) (Table 1). The L > N-channel dependence of NgCAM-mediated calcium entry was paralleled by a similar pattern of inhibition of anti-NgCAM-induced calcium entry by nifedipine and ω -conotoxin, at least at the doses of these inhibitors that we used (Table 2).

NgCAM Protein Was Not Itself a Trophic Stimulus

The time period during which NgCAM and its antibody elicited their respective calcium responses *in vitro* corresponded to the postmitotic age at which newly generated neurons leave the SZ to enter the brain paren-

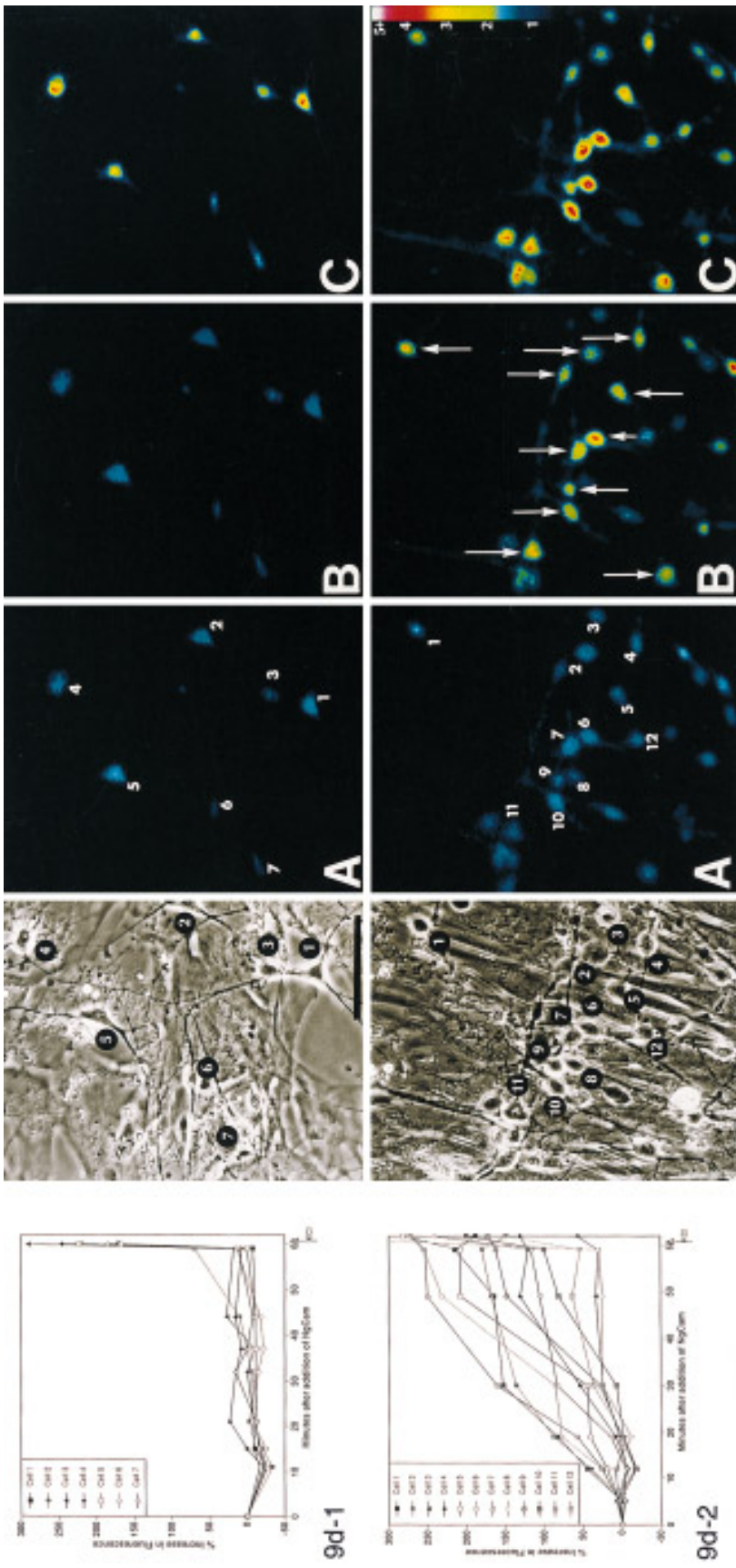


FIG. 4. Neuronal calcium responses to NgCAM were largely limited to bipolar cells. This figure compares the calcium responses of two fields after 9 DIV. At this time point, new neurons in these SZ cultures are pleomorphic; many still appear bipolar and relatively immature, while others have achieved tri- and multipolar morphologies. At this time point, neuronal calcium responses to NgCAM began to abate, concurrent with morphological maturation. In the top, a field of relatively mature tripolar neurons had no demonstrable calcium increments over baseline (A) to NgCAM (B), despite rapid and substantial calcium increments to depolarization (C; 60 mM KCl). In the bottom, another field in the same culture that included less morphologically mature cells, virtually all of the bipolar neurons responded to NgCAM with significant calcium increments (B, arrows); these bipolar neurons also displayed marked calcium elevations to KCl (C).

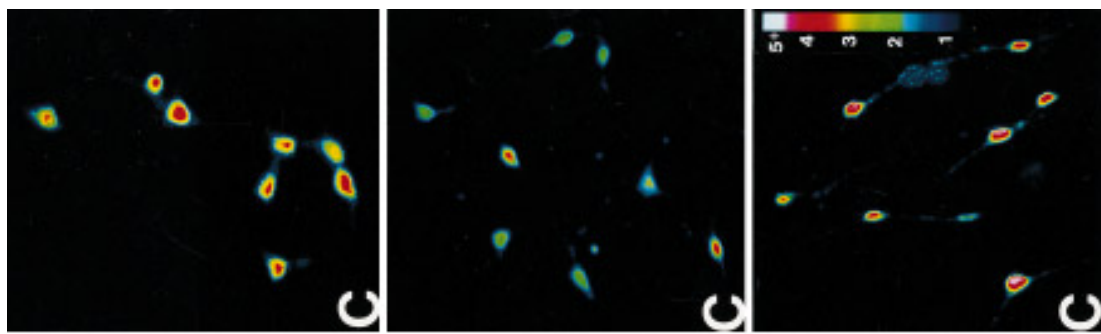
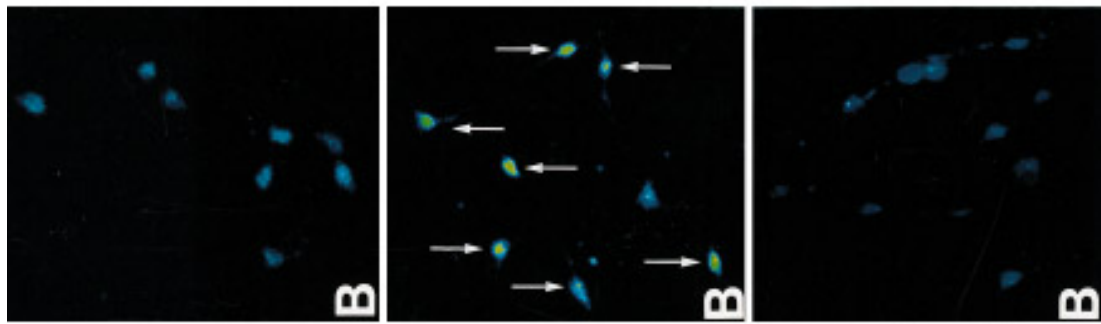
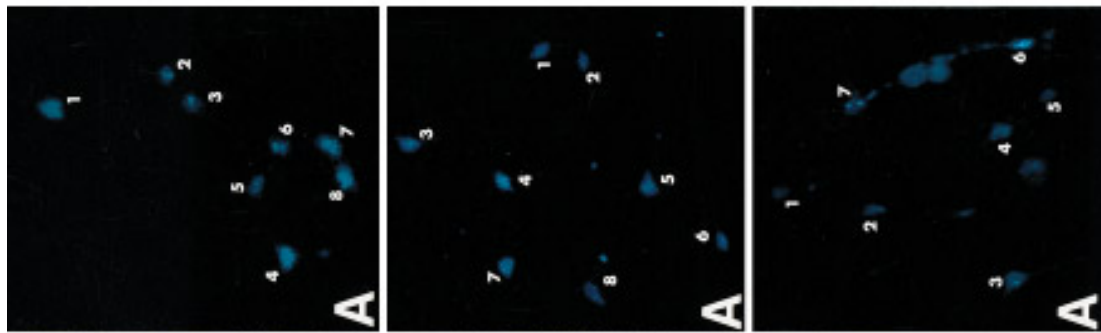
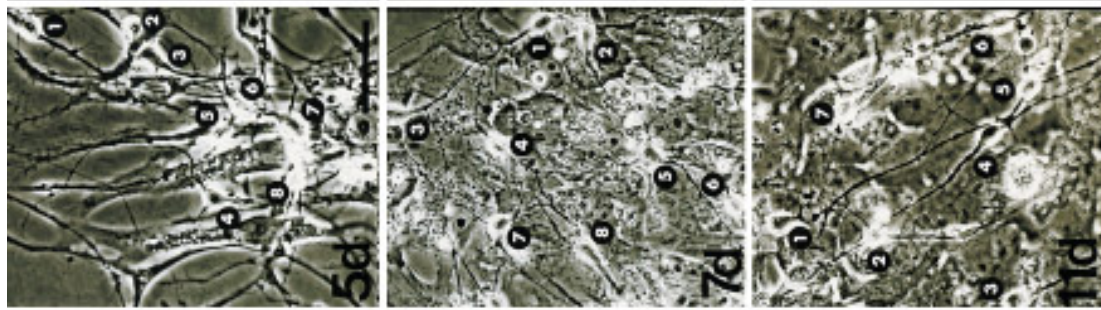
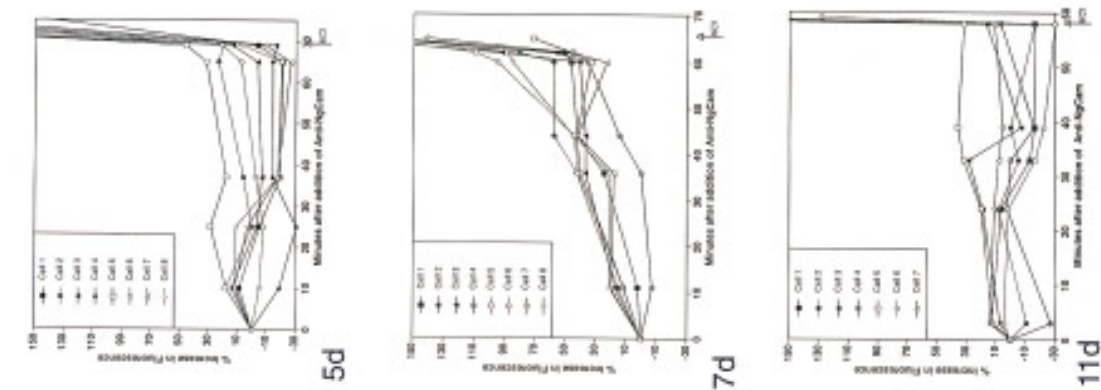


FIG. 6. The neuronal calcium response to anti-NgCAM Ig paralleled that to NgCAM itself. Neurons displayed little calcium response to anti-NgCAM IgG at 5 DIV (top row), but substantial responses at 7 DIV (middle row) and 9 DIV (not shown; see Table 2). By 11 DIV, however, the neuronal calcium responses to anti-NgCAM had fallen to unappreciable levels (bottom row). Again, in each row, A represents the baseline, B the maximal response of fluorescent neurons to anti-NgCAM during the first hour after exposure, and C, the response of these neurons to a depolarizing stimulus of 60 mM KCl. Thus, the time course of the neuronal calcium response to anti-NgCAM antibody corresponded to that of the calcium response to NgCAM protein, suggesting a common transduction pathway for calcium signaling to each.

TABLE 2

Effect of Anti-NgCAM IgG upon Ca_i^{2+} in Adult SZ-Derived Neurons

Condition	DIV	Cells observed ^a	% Neurons with Ca rise >20%	Average maximum Ca rise (%) ^b
As a function of the number of days of neuronal maturation <i>in vitro</i>				
Anti-NgCAM IgG (100 μg)	5	64	27	8 \pm 5
Anti-NgCAM	7	145	81	73 \pm 14*
Anti-NgCAM	9	63	75	74 \pm 18*
Anti-NgCAM	11	59	14	-11 \pm 5
In the presence of low calcium or calcium channel blockade				
Anti-NgCAM + Ca-free media ^c	7	64	22	-1 \pm 6**
Anti-NgCAM + nifedipine ^d	7	80	14	-5 \pm 5**
Anti-NgCAM + ω -conotoxin ^e	7	68	40	22 \pm 6**

^a As in Table 1, cells were sampled from an average of 3 cultures/treatment (range: 2–4 plates/group).

^b The average maximal % Ca increment of all neurons tested in each group \pm SE.

^c Calcium-free HBSS with 3 mM Mg, 2 mM EGTA.

^d Nifedipine (10 μM , Sigma) added 10 min before antibody addition.

^e ω -Conotoxin (1 μM , Gibco) added 10 min before antibody addition.

* Significant increase in Ca_i^{2+} relative to vehicle control, to $P < 0.05$.

** Significant inhibition in Ca_i^{2+} rise relative to anti-NgCAM alone at 7 DIV; also $P < 0.05$. Comparison of 5-, 7-, 9-, and 11-DIV anti-NgCAM and control additions by one-way ANOVA with post hoc Bonferroni *t* tests.

chyma *in vivo* (Barami et al., 1995). Thus, the departure of new neurons from the adult SZ 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. 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 SZ.

We therefore next asked whether the calcium signal elicited by NgCAM protein was directly neurotrophic. To this end, we added exogenous NgCAM antigen bidaily over the time span of 5–12 DIV (2.4 $\mu\text{g}/\text{plate}$) and assessed neuronal survival and maturation in the NgCAM-exposed cultures relative to matched controls given 2% BSA. Among 665 new neurons in 12 cultures exposed to NgCAM, 131 (19.7%) survived at 12 DIV, while among their controls ($n = 10$), 117 of 568 (20.6%) neurons survived ($P = 0.36$). Thus, NgCAM protein influenced neither neuronal survival nor maturation in these SZ explant outgrowths. This was puzzling given the impaired neuronal survival that we previously noted following anti-NgCAM exposure (Barami et al., 1994). Yet whereas the calcium responses of neurons exposed to anti-NgCAM antibody at 7 DIV were generally sustained, the calcium increments exhibited by these neu-

rons to NgCAM protein were typically transient and of lesser degree (data not shown).

Anti-NgCAM-Associated Neuronal Death Required Antibody-Induced Calcium Influx

We therefore next asked whether it was the antibody-induced calcium influx that caused the death of new neurons exposed to anti-NgCAM. We first confirmed the lethality of anti-NgCAM IgG exposure: Among 860 neurons ($n = 4$ explant outgrowths) exposed continuously to 100 μg anti-NgCAM IgG for 48 h beginning at 7 DIV, only 50 \pm 12% survived at 9 DIV. Control plates exposed to 100 μg preimmune IgG showed no such loss; their neuronal numbers actually increased from 7 to 9 DIV, by an average of 20 \pm 20%. To block the calcium increments associated with anti-NgCAM, we then exposed a sample of 372 neurons ($n = 4$ explants) to 100 $\mu\text{g}/\text{ml}$ anti-NgCAM and 10 mM nifedipine together, also over the span of 7–9 DIV. We found that the deleterious effect of anti-NgCAM exposure was largely prevented by nifedipine, such that 95 \pm 7% of the neurons so treated survived at 9 DIV, in contrast to the 50 \pm 12% neuronal survival in anti-NgCAM alone ($P = 0.013$). Thus, L-channel blockade with nifedipine prevented anti-NgCAM-mediated Ca entry and late neuronal death in tandem (Table 3). Since the anti-NgCAM-associated death of

TABLE 3

Effect of Anti-NgCAM IgG upon the Survival of Adult SZ-Derived Neurons

Treatment (continuous from 7–9 DIV)	Explants (n)	Baseline cell count (at 7 DIV)	% Survival ^a (at 9 DIV)
Control (preimmune sera)	3	490	112 ± 10
Anti-NgCAM IgG, 100 µg/ml	4	860	50 ± 12***
Nifedipine, 10 µM	4	865	79 ± 9
Anti-NgCAM + nifedipine	4	372	95 ± 13

^a Mean ± SE.** Different from control, $P = 0.009$. No other significant differences after Bonferroni adjustment for multiple comparisons.*** $F = 9.20$; $P = 0.0025$ by single-factor ANOVA.

these new neurons was calcium-dependent, it might have been an artifact of the relatively high and sustained calcium levels effected by antibody exposure: Calcium-associated neuronal death is a tandem function of both the quantitative extent and duration of cytosolic calcium elevation (Nedergaard *et al.*, 1991).

Absolute Neuronal Cytosolic Calcium Levels Did Not Differ from 5 through 11 DIV

Ratio measurements of fura-2 fluorescence were performed to quantify the absolute levels of intracellular calcium in both resting and NgCAM-stimulated neurons. Explant cultures of the adult finch SZ were raised on fibronectin or laminin/polylysine-coated coverslips, and examined at 5, 7, and 11 DIV. Ratio imaging of the fura-2 signal revealed that the baseline calcium levels of neurons in 5 (42 ± 2.1 nM), 7 (35 ± 2.1 nM), and 11 DIV (34 ± 5.7 nM) outgrowths did not differ significantly from one another (Table 4). Thus, in the absence of added

NgCAM, the basal calcium levels of adult SZ-derived neurons did not change significantly over the period spanning 5–11 DIV (Fig. 7).

Ratio Imaging Confirmed the Developmental Restriction of NgCAM-Induced Calcium Signaling

Using fura-2, we found that the responses of neurons to 1.2 µg NgCAM (2 µg/ml) at 5, 7, and 11 DIV paralleled those noted using fluo-3: At 7 DIV, neuronal calcium rose from 35 ± 2.1 nM to 88 ± 6.3 nM within an hour after addition of 1.2 µg NgCAM ($P < 0.01$ by two-way ANOVA, with post hoc Bonferroni t tests). In contrast, no such neuronal calcium increment was noted to NgCAM in the 5- or 11-DIV cultures (Table 4), verifying the absence of any detectable rise in the neuronal fluo-3 signal at these same time points (Table 1). In addition, vehicle addition yielded no increase in the fura-2 signal in the hour thereafter, at either 7 or 11 DIV (Table 4). Thus, the results obtained by confocal imaging of the fluo-3 signal were confirmed using the absolute measurements obtained by ratio imaging the fura-2 signal.

TABLE 4

Ratio Imaging with Fura-2 Confirmed the Developmental Restriction of NgCAM-Induced Calcium Signaling

Age (DIV)	Ca _i ²⁺ baseline	Treatment	Ca _i ²⁺ max after treatment	n, neurons
5	42 ± 2.1	NgCAM ^b	50 ± 2.3	82
7	35 ± 2.1	NgCAM	88 ± 6.3*	65
11	34 ± 5.7	NgCAM	37 ± 5.3	41
7	41 ± 12.1	Vehicle ^c	45 ± 13.3	12
11	39 ± 10.4	Vehicle	42 ± 11.1	14

Note. All values ± SE.

^a The maximum neuronal calcium level achieved within the first hour after exposure.^b 1.2 µg NgCAM.^c HBSS.* Higher than responses to vehicle and to NgCAM at other time-points, $P < 0.01$.

DISCUSSION

In this study, we found that new neurons generated from the SZ of the adult songbird brain responded to NgCAM with significant, sustained yet reversible increments in cytosolic calcium. NgCAM-elicited calcium signals were developmentally restricted, only being manifested by neurons between 6 and 9 DIV. In addition, these NgCAM-induced calcium increments were limited largely to bipolar migrants and were not exhibited by their postmigratory counterparts. Interestingly, the period during which NgCAM elicited a calcium response among new neurons *in vitro* corresponded to the postmitotic age at which neurons leave the adult SZ to enter

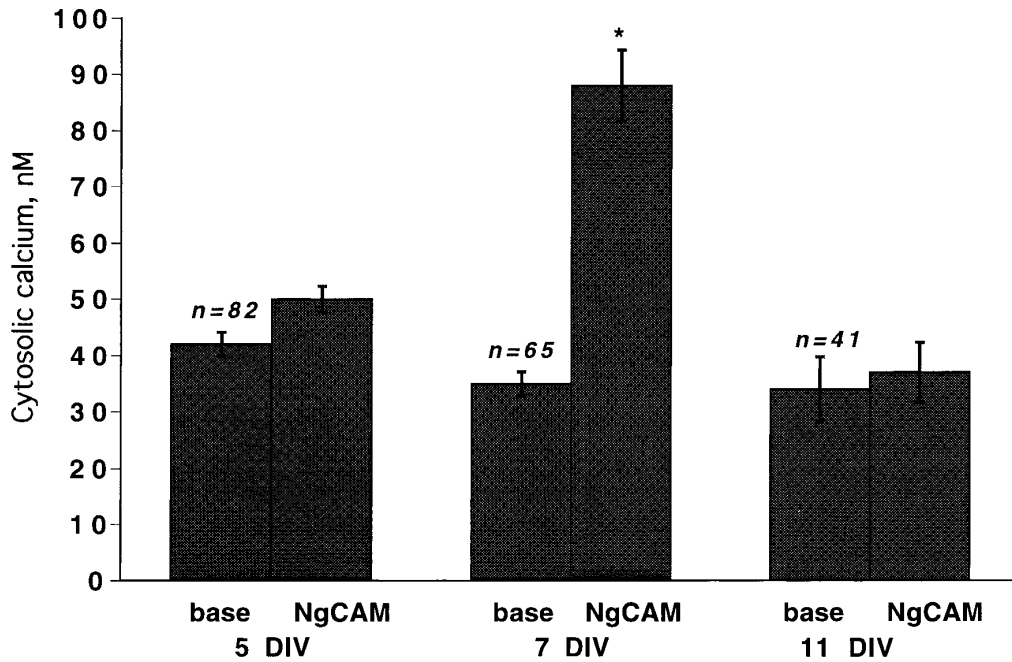


FIG. 7. Resting calcium levels did not vary as a function of postmitotic age, but were elevated specifically at 7 DIV by NgCAM. Because fluo-3 does not significantly alter its emission wavelength upon calcium binding, it cannot be used for ratio determinations of absolute calcium levels. To establish whether the *baseline* calcium levels of SZ-derived neurons differed after 5, 7, and 11 DIV, we used ratio imaging with fura-2 to quantify both the absolute neuronal calcium and the calcium response to NgCAM, at each of these time points. We found that the baseline level of cytosolic calcium did not differ significantly among 5, 7, and 11 DIV SZ-derived neurons. Furthermore, the calcium responses to NgCAM detected by fura-2 paralleled those noted using fluo-3: At 7 DIV, neuronal calcium rose significantly within an hour after addition of 2 μ g NgCAM, while no such calcium increment to NgCAM was noted in the 5- or 11-DIV cultures. *Denotes significant difference at $P < 0.01$.

the brain parenchyma *in vivo* (Barami et al., 1995). Indeed, throughout this same time period, anti-NgCAM IgG disrupted the migration and later survival of these neurons in culture (Barami et al., 1994). Thus, the down-regulation of the neuronal calcium response to NgCAM might represent a developmental switch, marking if not dictating the transition from migratory blast to young parenchymal neuron. Taken together, our results suggest a dynamic role for NgCAM in eliciting changes in cytosolic calcium which may be necessary for neuronal migration or survival during migration.

Role of NgCAM-Dependent Calcium Signaling during Neuronal Migration

In a previous study (Barami et al., 1994), we found that antibodies to NgCAM blocked the migration of adult SZ-derived neurons in culture, and that the survival of these neurons was greatly impaired after antibody exposure. Yet in our present study, added NgCAM protein did not exert any direct neurotrophic effect. Rather, a threshold degree of NgCAM-dependent calcium elevation, which

occurred only during a restricted period of neuronal ontogeny, appeared to be required for neuronal survival *in vitro*. These results suggested the possibility that NgCAM-dependent calcium signaling might act to promote neuronal survival during early migration.

The transient NgCAM-dependent calcium signaling exhibited by these cells during initial migration may contribute to the calcium-dependence of neuronal migration previously noted by Komuro and Rakic (1992, 1993). Although the basis for the latter observation remains unclear, an array of calcium-dependent tyrosine kinases are influenced by cellular calcium levels (Clapham, 1995). Enzymes such as proline-rich tyrosine kinase-2 (Lev et al., 1995) might provide direct links between calcium signals and the MAP kinase pathway, thereby relaying signals dictating transcriptional activation from the cell surface to the nucleus (Hill and Treisman, 1995). In addition, the NgCAM-dependent alteration in calcium homeostasis effected by NgCAM, or by other cognate heterophilic ligands in the extracellular milieu, might influence the new neuron's threshold to concurrent activation by humoral agents, such as the neurotrophins (Koike and Ta-

naka, 1991). In this regard, while the 153% rise in cytosolic calcium effected by NgCAM at 7 DIV was significant (Table 4), it paled in comparison to the depolarization-induced calcium increments measured in the same cells at the conclusion of each experiment: At 7 DIV, these neurons responded to 60 mM KCl with a mean calcium rise to 504 ± 58.6 nm, a >13-fold increase. Thus, the calcium elevation effected by NgCAM exposure may constitute a resetting of the basal neuronal calcium level, by which the responsiveness of new neurons to other calcium-transmitted signals might be modulated as migration proceeds. Alternatively, NgCAM-linked signaling might yield not a tonic increase in neuronal calcium, but rather a series of localized and reversible calcium responses and cycling perturbations. In each migrating neuron, the unique temporal sequence and spatial pattern of NgCAM-linked calcium responses might then encode considerable information with regard to both the geographic coordinates and hospitality of the local environment.

Initiation of NgCAM-Related Signaling at the Neuronal Surface

NgCAM-mediated neuronal signal transduction might be mediated by the homophilic binding of neuronal NgCAM to the NgCAM of the subventricular neuropil, or through the heterophilic interaction of neuronal NgCAM with cognate receptors of both the neuropil and the ependymal/radial guide cell surface. In this regard, Doherty and Walsh and their colleagues have proposed that the neuronal calcium response to L1 might be initiated through the binding of neuronal L1 to the fibroblast growth factor receptor (FGF-R1) (Williams *et al.*, 1994a). This event may occur by virtue of a CAM recognition domain spanning amino acids 151–170 of FGF-R1, a region homologous to a portion of L1 lying between the third and fourth Ig domains (Hlavin and Lemmon, 1991). The interaction between L1 and FGF-R may occur *in cis*, in the plane of the neuronal membrane, which would allow the calcium response to L1 to be initiated by the intrinsic tyrosine kinase of the L1-activated FGF-R1. Alternatively, neuronal L1 might bind *in trans* to the FGF-R1 of an adhesion-competent neuronal binding partner, such as the radial cell, with the neuronal calcium response being transduced by L1-associated cytoplasmic tyrosine kinases (see below; Ignelzi *et al.*, 1994). Indeed, FGF-R1 is widely expressed by cells in the SZ, opening the possibility of a heterophilic interaction between the NgCAM of newly migratory adult neurons and the FGF-R1 of their ependymal/radial cell binding partners. This possibility is particularly intriguing in light of the prefer-

ential association of adult SZ-derived neurons with ependymal cells *in vitro* (Goldman *et al.*, 1993a).

Additional mechanisms, other than FGF-R-dependent tyrosine kinase activation, might also mediate NgCAM/L1-dependent calcium signaling. Recent studies have suggested that the cytoplasmic tyrosine kinases might also serve this purpose. In particular, Maness and colleagues have demonstrated a role for *c-src* in L1-dependent neuritic outgrowth by cerebellar neurons (Atashi *et al.*, 1992; Ignelzi *et al.*, 1994). Presently, however, the means by which such cytoplasmic tyrosine kinases might associate with NgCAM/L1 remain unknown.

Intracellular Mechanisms of Calcium Signaling to NgCAM Activation

The existence of FGFR and *src*-dependent pathways of L1-dependent calcium signaling suggests that calcium entry to L1 might be regulated not by changes in the expression of L1, but rather by changes in the levels or activity of its downstream effectors. Whether through the FGFR or *src*-associated tyrosine kinases, the net effect of L1/NgCAM binding appears to be a rapid increment in neuronal cytosolic calcium. In our cultures, NgCAM appeared to activate both L- and N-type calcium channels, in that blockade of each attenuated a substantial proportion of the NgCAM-induced calcium influx (Table 1). Williams *et al.* (1994b) have demonstrated that L1-dependent neuritic extension can be mimicked by the action of arachidonic acid (AA), and that neuritic outgrowth in response to AA can be abolished by concurrent treatment with L- and N-channel antagonists. These findings suggest that the opening of L- and N-channels might require AA as a direct downstream effector of calcium channel activation. The NgCAM-driven pathway would then include the tyrosine kinase activation of membrane phospholipase C (PLC), itself dependent either in serial or in parallel upon pertussis-sensitive heterotrimeric G-protein, followed by the PLC-catalyzed production of diacylglycerol (DAG) and its subsequent conversion by DAG lipase to AA (Piomelli, 1993). This sequence of cellular events would be analogous to those previously identified in the signal transduction cascade implicated in L1-driven neuritic outgrowth by cerebellar granular cells (Williams *et al.*, 1994b).

Choice of Imaging Techniques

In this study, we looked at the responses of new neurons to NgCAM, largely as a function of neuronal age and migration state. To that end, the *differences* in the neuronal calcium levels elicited by NgCAM and its an-

tagonists were our experimental endpoints. We therefore chose to use as our principal calcium indicator dye fluo-3. However, fluo-3 does not significantly alter its emission wavelength upon calcium binding, so that it cannot be used for ratio determinations of absolute calcium levels. For this purpose, we used ratio imaging with fura-2, both to measure absolute neuronal calcium at 5, 7, and 11 DIV and to quantify the calcium response to NgCAM at each of these points. Nonetheless, fluo-3 has many advantages over ratio imaging with either fura-2 or fura's tetracarboxylate congeners, quin-2 and indo-1 (Minta *et al.*, 1989), so that we used fluo for most of our experiments. Specifically, the following considerations led us to choose fluo-3 for all of our *relative* calcium level determinations:

First, fluo-3 is visualized at longer wavelengths and with less intense excitation, and hence is associated with less phototoxicity upon repetitive exposure than fura. By virtue of its high quantum efficiency and dynamic range, fluo-3 can be imaged using less intense light than fura (Minta *et al.*, 1989). In addition, it is viewed under long wavelength excitation (488 nm), and hence is associated with little phototoxicity. This advantage was of particular concern given the relatively long observation periods (60–90 min) and recurrent laser exposures (every 5–10 min) of our experimental paradigm. These demands made the use of fura problematic, due to its requisite excitation by xenon ultraviolet light (UV) at relatively high intensity: Upon recurrent UV excitation, neurons may undergo a steady calcium influx, likely secondary to phototoxicity, that effectively precludes any hope of a stable calcium baseline upon which to assess treatment effects. Our use of fluo-3 obviated this concern to a large extent. During each of our experiments, we maintained a constant gain setting, and thereby could readily assess baseline shift; we noted little over the course of our 60- to 90-min runs.

Second, fura acts as a significant calcium buffer, and can substantially attenuate apparent free calcium levels and treatment-associated differences (Neher and Augustine, 1992). In fact, fura is able to buffer calcium so significantly as to compete successfully with endogenous calcium buffers; it has been found to artifactually minimize real changes in Ca during both calcium waves and subthreshold depolarization (Wagner and Keizer, 1994). Thus, fura can attenuate the very differences in calcium concentration that one hopes to measure. In contrast, fluo-3 has a lower K_d for calcium, and hence less capacity for calcium buffering, than fura. Since it does not perturb or attenuate free cytosolic calcium to the extent that fura does, fluo-3 is generally the more sensitive indicator for detecting manipulation-induced differences in calcium levels.

Third, fluo-3 loads better, at lower concentrations, than fura-2. We found that the adult SZ-derived neurons loaded well with micromolar concentrations of fluo-3, but required 10- to 100-fold higher concentrations of fura. The large amounts of fura needed for successful loading, taken together with its intrinsic calcium buffering capacity, might exacerbate further the artifactual suppression of calcium increments associated with fura. For all of these reasons, we reserved the use of fura to the quantification of absolute calcium levels at each time point, both at baseline and in response to NgCAM. In contrast, since the treatment-associated *difference* rather than the static *level* of calcium was more often the endpoint of interest in this study, we generally used fluo-3 to image these effects.

Overview

We have discovered a developmentally modulated coupling and uncoupling of NgCAM expression to NgCAM-dependent calcium signaling that occurs during the *in vitro* migration of new neurons from the adult avian SZ. This process represents a regulated and reversible coupling of a constitutively expressed adhesion molecule to a calcium-dependent signaling cascade. As such, it may represent a new strategy by which neuronal responses to environmental stimuli can be modified. In the adult avian SZ, the down-regulation of the neuronal calcium response to NgCAM after the first week *in vitro* suggests that NgCAM's function during initial neuronal cell body migration and parenchymal penetration is distinct from its role in later maturation and neuritic extension. This distinction may reflect the serial assumption of a variety of roles by NgCAM during neuronal ontogeny. It remains unclear if this process continues to be dynamically regulated upon neuronal maturation; conceivably, parenchymal neurons stably expressing NgCAM might reestablish NgCAM-dependent calcium signaling on an *ad hoc* basis, in response to environmental signals. Indeed, in light of the role of L1 in hippocampal long-term potentiation (Luthi *et al.*, 1994), it is reasonable to postulate that such NgCAM-linked calcium signaling might participate in information processing within the adult nervous system.

In the adult avian brain, the coupling of NgCAM to cellular signaling cascades might also be a target of hormonal or paracrine modulators of neuronal migration and survival. Neurogenic regions of the adult avian brain are underlain by a layer of estrogen-receptive neurons (Gahr *et al.*, 1987; Gahr, 1990), and even though the new neurons themselves do not express estrogen receptor, their survival is strongly modulated by endogenous es-

estrogen levels (Hidalgo *et al.*, 1995). The estrogen-receptive subventricular cells might therefore serve as a "gate-keeper" layer, acting to modulate neuronal survival during migration (Goldman, 1995). Interestingly, the time course during which new neurons traverse this estrogen-receptive layer corresponds to that during which the cells exhibit NgCAM-linked calcium signaling (Barami *et al.*, 1995). On this basis, it seems reasonable to postulate that the development of NgCAM-induced calcium signaling by new adult avian neurons might be dependent upon estrogen or estrogen-induced neurotrophins. As such, the tonic neuronal calcium rise elicited by NgCAM might be a permissive factor for the survival of new neurons during their initial migration. Together, these findings indicate that the activation of neuronal NgCAM by cognate parenchymal ligands might constitute an important new regulatory step in adult neurogenesis, by which cellular survival and fate might be established during the migration of newly generated neurons.

EXPERIMENTAL METHODS

Adult SZ Explant Preparation

Explants were obtained from a total of 26 adult male zebra finches (*Poephilia guttata*). Cultures were prepared from the neostriatal SZ, both overlying and directly medial to nucleus HVC, as previously described (Goldman, 1990; Goldman *et al.*, 1992). Typically, 4–6 explant outgrowths per bird demonstrated sufficient neuronal outgrowth (≥ 30 neurons) for inclusion in the study. The data reported here were derived from a total of 80 explant outgrowths.

Antigen Challenge

Prior to NgCAM addition, anti-NgCAM challenge, or drug treatment, each finch SZ explant was exposed to a vehicle control (DMEM); a stable calcium baseline among cells within the selected field was assured for at least 15 min, before the test antigen was added. Matched controls that received vehicle in place of NgCAM were also run. 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 5, 7, 9, or 11 DIV.

Antibody Addition

Rabbit anti-zebra finch IgG was prepared by injecting an adult New Zealand rabbit with finch NgCAM pro-

tein, 700 μg in complete Freund's adjuvant, with three subsequent monthly booster injections of 50 μg each in incomplete Freund's. Rabbit serum was harvested at monthly intervals thereafter, and each bleed was screened for NgCAM immunoreactivity using both immunocytochemistry and Western blot. Sera judged to have high titers of anti-NgCAM activity ($> 1:1000$ by each technique, using chick forebrain neurons as antigenic targets) were subjected to IgG precipitation with ammonium sulfate. The IgG was dialyzed overnight against HBSS and then redialyzed against DMEM/F12. Its final concentration in the dialysate was 4 mg/ml, and it was added to cultures at 100 $\mu\text{g}/\text{ml}$. In a previous study (Barami *et al.*, 1994), we found this to be an effective dose for the inhibition of neuronal outgrowth from these explants. In the same study, we also found no significant difference between the effects of anti-NgCAM IgG and Fab fragments prepared from this IgG and so chose to use the former for the present study.

Calcium Imaging by Confocal Microscopy

Each culture was preloaded with 10 μM fluo-3 aceto-methoxyester (fluo-3 AM, Molecular Probes) 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 6.03 (BioRad) and recorded on a Panasonic TQ-2028F optical disc recorder. At the completion of each experiment, cultures were challenged with 60 mM K^+ , to assess the integrity of their calcium responses to depolarization. Relative changes in fluorescence were then calculated and normalized against baseline fluorescence by $\Delta F/F$ (Nedergaard, 1994; Kirschenbaum *et al.*, 1994). Background counts were subtracted from all experiments, each of which was carried out at 25°C in HBSS.

Ratio Imaging of Intracellular Free Calcium

Ratio measurements of fura-2 fluorescence were performed to quantify the absolute levels of intracellular calcium in both resting and NgCAM-stimulated neurons. Explant cultures of the adult finch SZ were raised on fibronectin or laminin/polylysine-coated coverslips and examined at 5, 7, and 11 DIV. The cultures were loaded with 1 μM fura-2 AM in their culture medium for 30 min at 37°C, then washed with HEPES-buffered Hanks'

buffered salt solution (10 mM; pH 7.3), and incubated for another 20 min at 37°C in the absence of dye to allow complete deesterification of the fura-2 AM ester. The culture-bearing coverslips were then mounted in a Leiden chamber (Medical Systems, Inc.), at room temperature. The fura-2 signal was viewed with a 20× 0.75 N.A. fluo-rite objective, using an Olympus IX70 inverted microscope. A 200-W xenon/mercury lamp (OptiQuip) was used as light source. A filter wheel (Lambda-10, Sutter Instrument Comp.) rotated excitation filters (340 ± 5 nm and 380 ± 6.5 nm, Omega Optical) into the light path, while an MIT SIT68 camera (Dage Instruments) recorded the 510-nm fura-2 fluorescence emission. Universal Imaging software (Image-1/F) was used for hardware control and analysis.

Measurement of the fluorescence signal in terms of free Ca_i^{2+} was based upon the procedure described by Grynkiewicz *et al.* (1985). R_{min} and R_{max} were found to be 0.24 and 3.22 in our system, and a $K_d = 225 \times 10^{-9}$ M was used. For each measurement, the background signal was obtained by determining the emission signal in a representative field of the same size in an unloaded sister culture; this background was then subtracted from each experimental measurement.

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