HHS Public Access

Author manuscript

Nat Neurosci. Author manuscript; available in PMC 2011 February 01.

Published in final edited form as:

Nat Neurosci. 2010 August ; 13(8): 944–950. doi:10.1038/nn.2582.

cJun phosphorylation integrates calcium spike activity and *tlx3* expression to regulate neurotransmitter specification

Kurt W. Marek[1,*], **Lisa M. Kurtz**[1,2,*], and **Nicholas C. Spitzer**[1]

[1]Neurobiology Section, Division of Biological Sciences and Center for Molecular Genetics, Kavli Institute for Brain and Mind, University of California San Diego, La Jolla, California 92093-0357, USA.

[2]Neurosciences Graduate Program, University of California San Diego, La Jolla, California 92093-0662, USA.

Abstract

Neuronal differentiation is accomplished through cascades of intrinsic genetic factors initiated in neuronal progenitors by external gradients of morphogens. Activity was thought to be important only late in development, but recent evidence indicates that activity also regulates early neuronal differentiation. Activity in post-mitotic neurons prior to synapse formation can regulate phenotypic specification, including neurotransmitter choice, but the mechanisms are not clear. Here we identify a mechanism that links endogenous calcium spike activity with an intrinsic genetic pathway to specify neurotransmitter choice in neurons in the dorsal embryonic spinal cord of *Xenopus tropicalis*. Early activity modulates transcription of the GABAergic/glutamatergic selection gene *tlx3* and requires a variant cAMP response element (CRE) in its promoter. The cJun transcription factor binds to this CRE site, modulates transcription, and regulates neurotransmitter phenotype through its transactivation domain. Calcium signals through cJun N-terminal phosphorylation, thus integrating activity-dependent and intrinsic neurotransmitter specification. This mechanism provides a basis for early activity to regulate genetic pathways at critical decision points, switching the phenotype of developing neurons.

Excitability regulates many aspects of neuronal development in addition to forming the basis for rapid signaling in the mature nervous system 1,2. Key features determining neuronal structure and function are regulated by calcium (Ca) signaling, including cell proliferation and migration, axon guidance, synapse refinement, cell survival and neurotransmitter specification 3–9. Much work has been done to elucidate the way in which synaptic activity regulates these processes through neuronal gene expression 10,11, but less is known about modulation of neuronal development by earlier forms of activity prior to synapse formation.

Correspondence and requests for materials should be addressed to K.W. M. (kmarek@ucsd.edu).

*These authors contributed equally to this work.

Note: Supplementary information is available on the Nature Neuroscience website.

Competing interests statement The authors declare that they have no competing financial interests.

Here we focus on understanding the signaling cascade that mediates Ca spike-dependent specification of neurotransmitter phenotype in embryonic *Xenopus* spinal neurons.

Specification of neurotransmitters is a crucial aspect of development because it determines the polarity of synapses and the function of microcircuits. Many studies of neurotransmitter specification have focused on transcription factor cascades and downstream gene regulation. Gradients of members of the Shh and Bmp signaling pathways define the dorsoventral axis in the spinal cord and the expression of a combinatorial code of transcription factors that determine cell identity in neuronal progenitors 12,13. As neuronal precursors exit the cell cycle this combinatorial code initiates a cascade of transcription factors that determine the expression of effector genes and launch various programs underlying a neuron's functional identity, including axon trajectory, neurotransmitter specification and neurotransmitter receptor expression 14–18. Compelling evidence identifies roles for transcription factors in neurotransmitter choice. Ectopic expression of the homeobox gene MNR2 in neural cells can cause motor neuron differentiation, including the expression of acetylcholine 18. Dbx1 knockout mice exhibit an increase in GABA-positive interneurons 19. Gain- and loss-of-function experiments in mice show that transcription factors play a role in the expression of other classical neurotransmitters, glutamate and glycine 20–22. The central role played by transcription factors in neurotransmitter specification was demonstrated by the ability of Tlx3 and Lbx1 to act as switches in determining glutamatergic and GABAergic phenotypes in chick and mouse 23,24.

Activity also regulates neurotransmitter specification, including acetylcholine in mouse hypothalamic neurons 25 and dopamine in mouse petrosal ganglion neurons 26. In *Xenopus*, Ca spike activity regulates dopaminergic specification in the ventral suprachiasmatic nucleus and specification of four classical neurotransmitters, GABA, glutamate, glycine, and acetylcholine, in the spinal cord, via mechanisms that require transcription 9,27,28. Prior to synapse formation, the frequency of Ca spikes, which involve developmentally transient Ca-dependent action potentials and Ca-induced Ca release, homeostatically regulates specification of neurotransmitters in the spinal cord during a brief critical period 9,29. When Ca spikes are suppressed using molecular or pharmacological approaches, fewer neurons express the excitatory neurotransmitters glutamate and acetylcholine. In contrast, when Ca spiking is increased, more neurons express the inhibitory neurotransmitters GABA and glycine.

Here we provide evidence for a molecular mechanism by which Ca spikes during the critical period cause a change in the neuron's intrinsic transcriptional cascade specifying GABA and glutamate. The end result is the mature neurotransmitter phenotype of the neuron, determined by both genetic factors and activity. This interplay of intrinsic cellular context and Ca signaling is likely to allow assembly of circuits with functions appropriate to an environment of changing activity.

RESULTS

Activity and *tlx3* interact to specify neurotransmitter fate

To discover possible integration points between early activity and genetic pathways regulating neuronal differentiation we sought to identify genes with activity-dependent expression by *in situ* hybridization for transcription factors involved in neurotransmitter specification. *Xenopus tropicalis* (*X. tropicalis*) embryos (Supplementary Fig. 1) 30 were injected in one cell at the two-cell stage with mRNA encoding myc-tagged K_{ir} to suppress Ca spike activity unilaterally 9. Overexpression of this inward rectifier potassium channel hyperpolarizes neurons and suppresses generation of Ca spikes. Ca spike suppression caused an increase in both the area and intensity of expression of the t-cell leukaemia homeobox-3 (*tlx3*) gene compared to the unmanipulated side of the embryo (Supplementary Fig. 2). *tlx3* expression begins in post-mitotic lateral spinal neurons at the neural tube stage. These neurons migrate dorsally and medially, strongly suggesting they are glutamatergic Rohon-Beard sensory neurons and dorsolateral interneurons. *tlx3* expression continues throughout the critical period for neurotransmitter specification when spontaneous Ca spike activity is present (Supplementary Fig. 3) 31. To manipulate *tlx3* expression we generated splice- and translation-blocking morpholinos (MOs; Supplementary Fig. 4) and an overexpression construct. Knockdown of *tlx3* function with MOs caused an increase in the number of GABA-immunoreactive (-IR) neurons and a decrease in the number of vesicular glutamate transporter (VGluT1)-IR neurons in the spinal cord, demonstrating that *tlx3* functions as a switch specifying the glutamatergic over the GABAergic phenotype as in mouse and chick 23,24. Overexpression of *tlx3* caused the opposite effect on neurotransmitter specification. In addition, the overexpression construct lacks the target sequence for the translation-blocking MO and reversed the MO phenotype when they were co-expressed (Supplementary Fig. 5). Antagonism of *lbx1* by *tlx3* determines the glutamatergic over GABAergic phenotype in mice 24. Although *lbx1* is not co-expressed with *tlx3* in Rohon-Beard cells, *tlx3* MOs reduced VGluT1 expression in these sensory neurons, demonstrating that *lbx1* is dispensable for the glutamatergic phenotype in these cells (Supplementary Fig. 5). *tlx1* partially compensates for the loss of *tlx3* in mice 24. Similar to mice, *tlx1* is expressed in a subset of neurons expressing *tlx3* (data not shown), which may account for the VGluT1-IR cells remaining after *tlx3* knockdown.

To determine whether *tlx3* is an integration point for activity and a genetic pathway specifying neurotransmitter expression, we performed simultaneous manipulations of Ca activity and *tlx3* gene function. Overexpressing mRNA encoding K_{ir} to suppress Ca activity produced a decrease in the number of GABA-IR neurons and an increase in the number of VGluT1-IR neurons. MO knockdown of *tlx3* generated the opposite phenotype. Simultaneous suppression of Ca activity and knockdown of *tlx3* gene function phenocopied the result obtained by *tlx3* knockdown (Fig. 1a,b). Overexpressing mRNA encoding Na_v to increase sodium channel expression and enhance Ca activity 9 caused an increase in the incidence of GABA-IR neurons and a decrease in the incidence of VGluT1-IR neurons, while overexpressing *tlx3* produced the opposite phenotype. Simultaneous enhancement of Ca activity and overexpression of *tlx3* phenocopied overexpression (Fig. 1c,d). These results demonstrate that *tlx3* is required for activity-suppression-dependent respecification of

GABA and glutamate and that overexpression occludes the switch caused by increased Ca spike activity. Furthermore, these data indicate that Ca activity regulates neurotransmitter specification upstream of a crucial genetic choice point.

A variant CRE mediates activity-dependent *tlx3* transcription

The interaction between activity and *tlx3* in specifying neurotransmitter phenotype makes the regulation of this gene attractive for analysis of the integration of genetic factors and early activity-dependent processes controlling neuronal differentiation. Aligning human and mouse genomic sequences with more distantly related vertebrates has proven successful for identifying important cis-regulatory elements 32,33. We adopted this approach to identify elements that may be important for imparting activity dependence. Comparative genomics revealed that only a single region of the gene is conserved among *X. tropicalis*, mouse, and human for ~15 kb upstream and ~40 kb downstream of the gene (see Online Methods). 384 bp of the promoter region upstream of the start ATG is 65% conserved including a 152 bp subregion that is 81% conserved (Supplementary Fig. 6a). This 384 bp region drives eGFP expression in the dorsal spinal cord and sensory ganglia, similar to the endogenous expression pattern, demonstrating that it is sufficient to recapitulate native expression of *tlx3* (Supplementary Fig. 6b). In addition, expression is observed in dorsal muscle, indicating that the 384 bp region is likely missing an additional negative regulatory element that normally represses expression in this tissue. Because this small region is highly conserved among frog, mouse, and human, we surmised that it may contain an activity-responsive element.

To test this hypothesis, we generated a wildtype reporter construct to express the firefly luciferase gene under control of the conserved 384 bp of the promoter region. This reporter was co-injected with a normalization vector (see Online Methods) and mRNA encoding K_{ir} or Na_v to suppress or enhance Ca-activity, respectively. Suppressing Ca-activity increased expression of the luciferase reporter while enhancing Ca-activity decreased reporter expression (Fig. 2a). The smaller change observed when enhancing activity may result from asymmetric effects of spike suppression and enhancement on downstream regulatory elements. No changes in reporter expression were observed when assays were performed on larvae from which the neural tube had been dissected (Supplementary Fig. 6c), indicating that expression of luciferase in other embryonic tissue is not activity-dependent and that the activity-dependent changes observed are occurring in the neural tube. In addition, no changes in reporter expression were observed when luciferase assays were performed on embryos prior to the period of Ca activity responsible for regulating neurotransmitter specification, demonstrating that activity present during earlier periods of development does not influence reporter expression (Supplementary Fig. 6d).

These data demonstrate that transcriptional regulation of the genetic pathway by Ca spike activity could determine neurotransmitter specification and suggest that there may be activity-responsive elements within this promoter. Analysis of the 384 bp promoter region for conserved transcription factor binding sites identified a variant form of the cAMP response element (CRE; TGATGTCA), an activating protein-1 (AP1) site, a signal transducer and activator of transcription (STAT) site, in addition to two nuclear factor Y

(NFY) loci previously shown to be important for basal transcription of *tlx3* (Supplementary Fig. 6a) 34. The high conservation of this region between *X. tropicalis*, mouse, and human suggests the importance of these sites for regulation of *tlx3* transcription.

CRE and AP1 have both been implicated in activity-dependent transcriptional regulation 11,35. To determine the requirement of these sites for *tlx3* regulation by Ca activity, we performed additional luciferase assays with reporters containing either a mutated AP1 binding site ($AP1_{mut}$ reporter; Fig. 2b) or a mutated CRE (CRE_{mut} reporter; Fig. 2c). Mutating the AP1 site had no effect, while mutating the CRE abolished the activity-dependence of reporter expression. These data indicate that the CRE is required for activity-dependent regulation of *tlx3* and suggest that this site could integrate genetic and activity-dependent specification of glutamatergic and GABAergic phenotypes.

cJun regulates the intrinsic transmitter specification pathway

We tested the ability of candidate transcription factors to bind the CRE sequence in the *tlx3* promoter using electrophoretic mobility shift assays (EMSAs). Previous work indicates that cJun and ATF2 preferentially bind the variant CRE sequence TGATGTCA 36. Incubating probe containing the CRE and flanking sequence from the *tlx3* promoter with recombinant cJun or ATF2 caused a shift in migration of the probe that was blocked with excess unlabeled probe, but not by excess unlabeled probe containing a mutant CRE or a non-specific probe (Fig. 3a,b). Luciferase assays demonstrated that constitutively overexpressing a dominant negative mutant of cJun lacking the transactivation domain ($cJun_{TAM}$) 37, but not a similar dominant-negative $ATF2_{TAM}$, caused a CRE-dependent increase in the reporter (Fig. 3c). We confirmed the presence of cJun in the embryonic spinal cord by RT-PCR (Fig. 3d) and immunocytochemistry (Fig. 3e). These data suggest that cJun can act through the variant CRE site to regulate transcription of *tlx3*.

To avoid early developmental defects caused by constitutive manipulation of cJun with morpholinos, we generated hormone-inducible overexpression constructs for wildtype cJun and the dominant negative mutant $cJun_{TAM}$. These overexpressed fusion proteins remain inactive until injected embryos are incubated with dexamethasone 38. Inducing overexpressed wildtype cJun decreased wildtype reporter levels in the luciferase assay (Fig. 4a), while induction of $cJun_{TAM}$ increased wildtype luciferase reporter expression (Fig. 4b). The effects were abolished when the CRE site was mutated in the luciferase reporter (Fig. 4a,b). These experiments show that cJun can control *tlx3* transcription via the CRE binding site.

We next directly assessed the role of cJun in neurotransmitter specification by overexpressing wildtype or dominant negative $cJun_{TAM}$ and immunostaining for GABA and VGluT1. Inducing overexpressed cJun increased the number of neurons expressing GABA and decreased the number of neurons expressing VGluT1 (Fig. 4c,d), similar to the results obtained for *tlx3* knockdown. In contrast, inducing $cJun_{TAM}$ generated the opposite phenotype (Fig. 4c,d), similar to *tlx3* overexpression. Thus, both Ca activity and cJun regulate intrinsic neurotransmitter specification by modulating *tlx3* transcription via the CRE site located in its promoter.

Integration by cJun phosphorylation

To find out if cJun integrates genetic and activity-dependent neurotransmitter specification pathways we determined whether Ca spike activity regulates cJun function. The extent of cJun expression was unaffected by manipulation of Ca activity via unilateral injection of mRNA encoding K_{ir} or Na_v (Fig. 5a). Phosphorylation of S63, S73, T91 and T93 is the primary basis of regulating the transactivation domain of cJun 39. Because cJun regulation of *tlx3* was altered by deletion of the transactivation domain, we ascertained whether Ca activity regulates phosphorylation of amino acids in this region. Immunocytochemistry using phosphospecific antibodies showed that phosphorylation of S73 and T91 was decreased in response to Ca spike suppression and increased in response to Ca spike enhancement (Fig. 5b,c).

Mutations converting all four residues to alanines ($cJun_{mut}$) reproduced the CRE-dependent increase of *tlx3* reporter expression observed for the cJun transactivation domain mutant in luciferase assays (Fig. 6a). In addition, induction of $cJun_{mut}$ increased *tlx3* expression observed by *in situ* (Supplementary Fig. 7). Overexpressing this phosphorylation mutant caused a reduction in GABA-IR and an increase in VGlut1-IR (Fig. 6b,c). These experiments demonstrate the functional role of these phosphorylation sites and identify cJun phosphorylation as the Ca spike entry point in the genetic pathway.

DISCUSSION

Our results reveal a mechanism by which early activity and genetic factors interact to drive differentiation. The data support a model in which Ca spike activity prior to synapse formation modulates the genetic pathway for specification of neurotransmitter phenotype (Supplementary Fig. 8a). It is currently unclear whether Ca spike activity functions cell-autonomously. Ca spikes could cause phenotypic changes within the spiking cell or they could initiate a signaling cascade to neighbouring cells via diffusible factors. However, the downstream signaling cascade in dorsal neurons initiated by Ca activity is cell-autonomous -- it involves phosphorylation of cJun that regulates *tlx3* transcription through a CRE site in its promoter (Supplementary Fig. 8b). cJun appears to function as a repressor of *tlx3* transcription. Although cJun is often described as an activator, it functions as a repressor in some cases, operating by recruiting co-repressors to the promoter or sequestering other activation factors 40.

The conservation of the variant CRE site in *Xenopus*, mouse and human *tlx3* suggests that activity-dependent regulation of *tlx3* is present in mammals, as well as in *X. tropicalis*. However, there appear to be differences in the specification of glutamatergic and GABAergic neurons in *Xenopus* and mammals. In mice, *tlx3* functions to antagonize *lbx1*: in the absence of *tlx3* neurons default to a GABAergic fate under the control of *lbx1*, but when *lbx1* is also knocked out, the glutamatergic fate is restored 24. *tlx3* and *lbx1* appear to be largely non-overlapping in Rohon-Beard cells of *Xenopus tropicalis*, so this mechanism is not conserved. Another transcription factor may fulfill the same role as *lbx1* or *tlx3* may act alone in *Xenopus*. Although there is strong conservation in the promoter sequence of *tlx3* across species, other species-specific sites regulating activity-dependence are not excluded.

Strikingly, a strong cell-non-autonomous effect is also observed in both *tlx3* loss-of-function and cJun gain-of-function embryos: the increase in GABA-IR cells is greater than the loss of VGluT1-IR cells and there are additional ventral GABA-IR neurons in the *tlx3* morphants where *tlx3* is not expressed. This result raises the possibility that other mechanisms exist to maintain circuit homeostasis in the developing spinal cord, perhaps by altering neurotransmitter specification in downstream neurons or generating compensatory synaptic connections.

Many other transcription factors are involved in neurotransmitter specification, but their relation to activity-dependent processes is unclear. However, several examples of activity-dependent neurotransmitter regulation involve transcription. Changes in Ca spike activity change the number of neurons expressing tyrosine hydroxylase transcripts in the postembryonic brain 27. Synaptic activation leads to appearance of GAD67 and vesicular GABA transporter transcripts in adult glutamatergic granule cells and $GABA_A$ receptor-mediated responses to granule cell stimulation 41. These results suggest that the interplay between activity and genetic pathways in neurotransmitter specification is extensive. Indeed, the difference between the changes in VGluT1-IR and GABA-IR that we observe when manipulating activity is likely caused by the involvement of other transcription factors, which may or may not be differentially regulated by activity. Future work will determine whether the mechanisms underlying activity-dependent specification of other neurotransmitters share common elements.

This integration of activity and genetic pathways may have important implications for development of the nervous system. The Ca activity-dependent switch from excitatory glutamatergic to inhibitory GABAergic phenotype through regulation of a binary selection gene could reverse the polarity of a developing neuronal circuit. Our findings raise the intriguing possibility that activity-dependent regulation occurs at other fundamental choice points during development. Activity-responsive genes may identify developmental switches where extrinsic factors impinge on intrinsic pathways.

Activity plays an important role in many aspects of neuronal development. The functions and mechanisms of synaptic activity have been extensively studied. For example, this form of activity regulates the number and strength of synapses formed 42,43 and cell survival 44. Less is known about the function of earlier forms of activity, although it has been identified throughout the nervous system in many organisms. Early activity regulates proliferation of neuronal precursors and neuronal migration as well as neuronal differentiation 2. Ca transients occur at many times and in many places during development, but the mechanisms by which their patterns regulate differentiation have not been fully elucidated. It is unclear how Ca transients generated at frequencies of 1–20 hr^{-1} initiate the signaling cascade regulating neurotransmitter phenotype, although bursts of these Ca transients stimulate generation of cAMP transients 45. Coding of transcription by the frequency of Ca transients has been reported in T lymphocytes 46 and in basophilic leukaemia cells 47. Stimulation of CaM kinase II, NFAT and OCT/OAP transcription factor activity depends on relatively high frequencies of Ca transients 46–48. In contrast, activation of the NF-kB transcription factor by Ca oscillations occurs at low frequencies 46 that are within the range of those generated by developing *Xenopus* spinal neurons. It will be interesting to assess the role of cJun and

CRE in other organisms and to identify signaling components linking Ca spikes to changes in cJun activity. N-terminal Jun kinases regulate cJun activity via phosphorylation of S63/S73 and T91/T93 (39), making them attractive candidates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sera Chung, Gabriela Monsalve and Armando de la Torre for technical assistance. We thank Darwin Berg, Anirvan Ghosh and Joseph Gleeson for critical comments on the manuscript. This work was supported by a Damon Runyon Cancer Research Foundation Postdoctoral Fellowship to K.W.M., NIH Predoctoral Fellowship T32 AG00216 to L.M.K. and by NIH grant MH 074702 to N.C.S.

References

1. Goodman CS, Shatz CJ. Developmental mechanisms that generate precise patterns of neuronal connectivity. Cell. 1993; 72:77–98. [PubMed: 8428376]
2. Spitzer NC. Electrical activity in early neuronal development. Nature. 2006; 444:707–712. [PubMed: 17151658]
3. Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR. Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. Neuron. 2004; 43:647–661. [PubMed: 15339647]
4. Komuro H, Rakic PK. Intracellular Ca^{2+} fluctuations modulate the rate of neuronal migration. Neuron. 1996; 17:275–285. [PubMed: 8780651]
5. Hanson MG, Landmesser LT. Normal patterns of spontaneous activity are required for correct motor axon guidance and the expression of specific guidance molecules. Neuron. 2004; 43:687–701. [PubMed: 15339650]
6. Hanson MG, Landmesser LT. Increasing the frequency of spontaneous rhythmic activity disrupts pool-specific axon fasciculation and pathfinding of embryonic spinal motoneurons. J Neurosci. 2006; 26:12769–12780. [PubMed: 17151280]
7. Buffelli M, et al. Genetic evidence that relative synaptic efficacy biases the outcome of synaptic competition. Nature. 2003; 424:430–434. [PubMed: 12879071]
8. Yano S, Tokumitsu H, Soderling TR. Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature. 1998; 396:584–587. [PubMed: 9859994]
9. Borodinsky LN, et al. Activity-dependent homeostatic specification of transmitter expression in embryonic neurons. Nature. 2004; 429:523–530. [PubMed: 15175743]
10. Lin Y, et al. Activity-dependent regulation of inhibitory synapse development by Npas4. Nature. 2008; 455:1198–1204. [PubMed: 18815592]
11. Flavell SW, Greenberg ME. Signaling mechanisms linking neuronal activity to gene expression and plasticity of the nervous system. Ann Rev Neurosci. 2008; 31:563–590. [PubMed: 18558867]
12. Yamada T, Pfaff SL, Edlund T, Jessell TM. Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell. 1993; 73:673–686. [PubMed: 8500163]
13. Liem KFJ, Tremml G, Roelink H, Jessell TM. Dorsal differentiation of neural plate cells induced by BMP-mediated signals from epidermal ectoderm. Cell. 1995; 82:969–979. [PubMed: 7553857]
14. Thor S, Andersson SG, Tomlinson A, Thomas JB. A LIM-homeodomain combinatorial code for motor-neuron pathway selection. Nature. 1999; 397:76–80. [PubMed: 9892357]
15. Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet. 2001; 1:20–29. [PubMed: 11262869]
16. Baumgardt M, Miguel-Aliaga I, Karlsson D, Ekman H, Thor S. Specification of neuronal identities by feedforward combinatorial coding. PLoS Biology. 2007; 5:e37. [PubMed: 17298176]

17. Ma Q. Transcriptional regulation of neuronal phenotype in mammals. J Physiol (Lond). 2006; 575:379–387. [PubMed: 16825304]
18. Tanabe Y, William CM, Jessell TM. Specification of motor neuron identity by the MNR2 homeodomain protein. Cell. 1998; 95:67–80. [PubMed: 9778248]
19. Pierani A, et al. Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. Neuron. 2001; 29:367–384. [PubMed: 11239429]
20. Mo Z, Li S, Yang X, Xiang M. Role of the Barhl2 homeobox gene in the specification of glycinergic amacrine cells. Development. 2004; 131:1607–1618. [PubMed: 14998930]
21. Pillai A, Mansouri A, Behringer R, Westphal H, Goulding M. Lhx1 and Lhx5 maintain the inhibitory-neurotransmitter status of interneurons in the dorsal spinal cord. Development. 2007; 134:357–366. [PubMed: 17166926]
22. Mizuguchi R, et al. Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. Nat Neurosci. 2006; 9:770–778. [PubMed: 16715081]
23. Cheng L, et al. Tlx3 and Tlx1 are post-mitotic selector genes determining glutamatergic over GABAergic cell fates. Nat Neurosci. 2004; 7:510–517. [PubMed: 15064766]
24. Cheng L, et al. Lbx1 and Tlx3 are opposing switches in determining GABAergic versus glutamatergic transmitter phenotypes. Nat Neurosci. 2005; 8:1510–1515. [PubMed: 16234809]
25. Liu X, Popescu IR, Denisova JV, Neve RL, Corriveau RA, Belousov AB. Regulation of cholinergic phenotype in developing neurons. J Neurophysiol. 2008; 99:2443–2455. [PubMed: 18322006]
26. Brosenitsch TA, Katz DM. Expression of Phox2 transcription factors and induction of the dopaminergic phenotype in primary sensory neurons. Mol Cell Neurosci. 2002; 20:447–457. [PubMed: 12139921]
27. Dulcis D, Spitzer NC. Illumination controls differentiation of dopamine neurons regulating behaviour. Nature. 2008; 456:195–201. [PubMed: 19005547]
28. Gu X, Spitzer NC. Distinct aspects of neuronal differentiation encoded by frequency of spontaneous Ca^{2+} transients. Nature. 1995; 375:784–787. [PubMed: 7596410]
29. Root CM, Velázquez-Ulloa NA, Monsalve GC, Minakova E, Spitzer NC. Embryonically expressed GABA and glutamate drive electrical activity regulating neurotransmitter specification. J Neurosci. 2008; 28:4777–4784. [PubMed: 18448654]
30. Chang LW, Spitzer NC. Spontaneous calcium spike activity in embryonic spinal neurons is regulated by developmental expression of the Na^+, K^+-ATPase β3 subunit. J Neurosci. 2009; 29:7877–7885. [PubMed: 19535599]
31. Patterson KD, Krieg PA. Hox11-family genes XHox11 and XHox11L2 in *Xenopus*: XHox11L2 expression is restricted to a subset of the primary sensory neurons. Dev Dyn. 1999; 214:34–43. [PubMed: 9915574]
32. Nakano T, Windrem M, Zappavigna V, Goldman SA. Identification of a conserved 125 base-pair Hb9 enhancer that specifies gene expression to spinal motor neurons. Dev Biol. 2005; 283:474–485. [PubMed: 15913596]
33. Ovcharenko I, Stubbs L, Loots GG. Interpreting mammalian evolution using *Fugu* genome comparisons. Genomics. 2004; 84:890–895. [PubMed: 15475268]
34. Borghini S, Vargiolu M, Di Duca M, Ravazzolo R, Ceccherini I. Nuclear factor Y drives basal transcription of the human TLX3, a gene overexpressed in T-cell acute lymphocytic leukemia. Mol Cancer Res. 2006; 4:635–643. [PubMed: 16966433]
35. Sugiyama C, et al. Activator protein-1 responsive to the group II metabotropic glutamate receptor subtype in association with intracellular calcium in cultured rat cortical neurons. Neurochem Int. 2007; 51:467–475. [PubMed: 17559977]
36. Heckert LL, Schultz K, Nilson JH. The cAMP response elements of the alpha subunit gene bind similar proteins in trophoblasts and gonadotropes but have distinct functional sequence requirements. J Biol Chem. 1996; 271:31650–31656. [PubMed: 8940185]
37. Peng Y, et al. Neural inhibition by c-Jun as a synergizing factor in bone morphogenetic protein 4 signaling. Neuroscience. 2002; 109:657–664. [PubMed: 11927148]
38. Picard D. Posttranslational regulation of proteins by fusions to steroid-binding domains. Methods Enzymol. 2000; 327:385–401. [PubMed: 11044998]

39. Raivich G. c-Jun expression, activation and function in neural cell death, inflammation and repair. J Neurochem. 2008; 107:898–906. [PubMed: 18793328]
40. Ghosh S, Wu Y, Li R, Hu Y. Jun proteins modulate the ovary-specific promoter of aromatase gene in ovarian granulosa cells via a cAMP-responsive element. Oncogene. 2005; 24:2236–2246. [PubMed: 15688015]
41. Gómez-Lira G, Lamas M, Romo-Parra H, Gutiérrez R. Programmed and induced phenotype of the hippocampal granule cells. J Neurosci. 2005; 25:6939–6946. [PubMed: 16049169]
42. Flavell SW, et al. Activity-dependent regulation of MEF2 transcription factors suppresses excitatory synapse number. Science. 2006; 311:1008–1012. [PubMed: 16484497]
43. Lin Y, et al. Activity-dependent regulation of inhibitory synapse development by Npas4. Nature. 2008; 455:1198–1204. [PubMed: 18815592]
44. Hardingham GE, Fukunaga Y, Bading H. Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci. 2002; 5:405–414. [PubMed: 11953750]
45. Gorbunova YV, Spitzer NC. Dynamic interactions of cyclic AMP transients and spontaneous Ca^{2+} spikes. Nature. 2002; 418:93–96. [PubMed: 12097913]
46. Dolmetsch RE, Xu K, Lewis RS. Calcium oscillations increase the efficiency and specificity of gene expression. Nature. 1998; 392:933–936. [PubMed: 9582075]
47. Li W, Llopis J, Whitney M, Zlokarnik G, Tsien RY. Cell-permeant caged InsP3 ester shows that Ca2+ spike frequency can optimize gene expression. Nature. 1998; 392:936–941. [PubMed: 9582076]
48. De Koninck P, Schulman H. Sensitivity of CaM kinase II to the frequency of Ca^{2+} oscillations. Science. 1998; 279:227–230. [PubMed: 9422695]
49. Sive, HL.; Grainger, RM.; Harland, RM. Early Development of Xenopus laevis: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 2000.
50. Ovcharenko I, Nobrega MA, Loots GG, Stubbs L. ECR Browser: a tool for visualizing and accessing data from comparisons of multiple vertebrate genomes. Nucleic Acids Res. 2004; 32:W280–W286. [PubMed: 15215395]

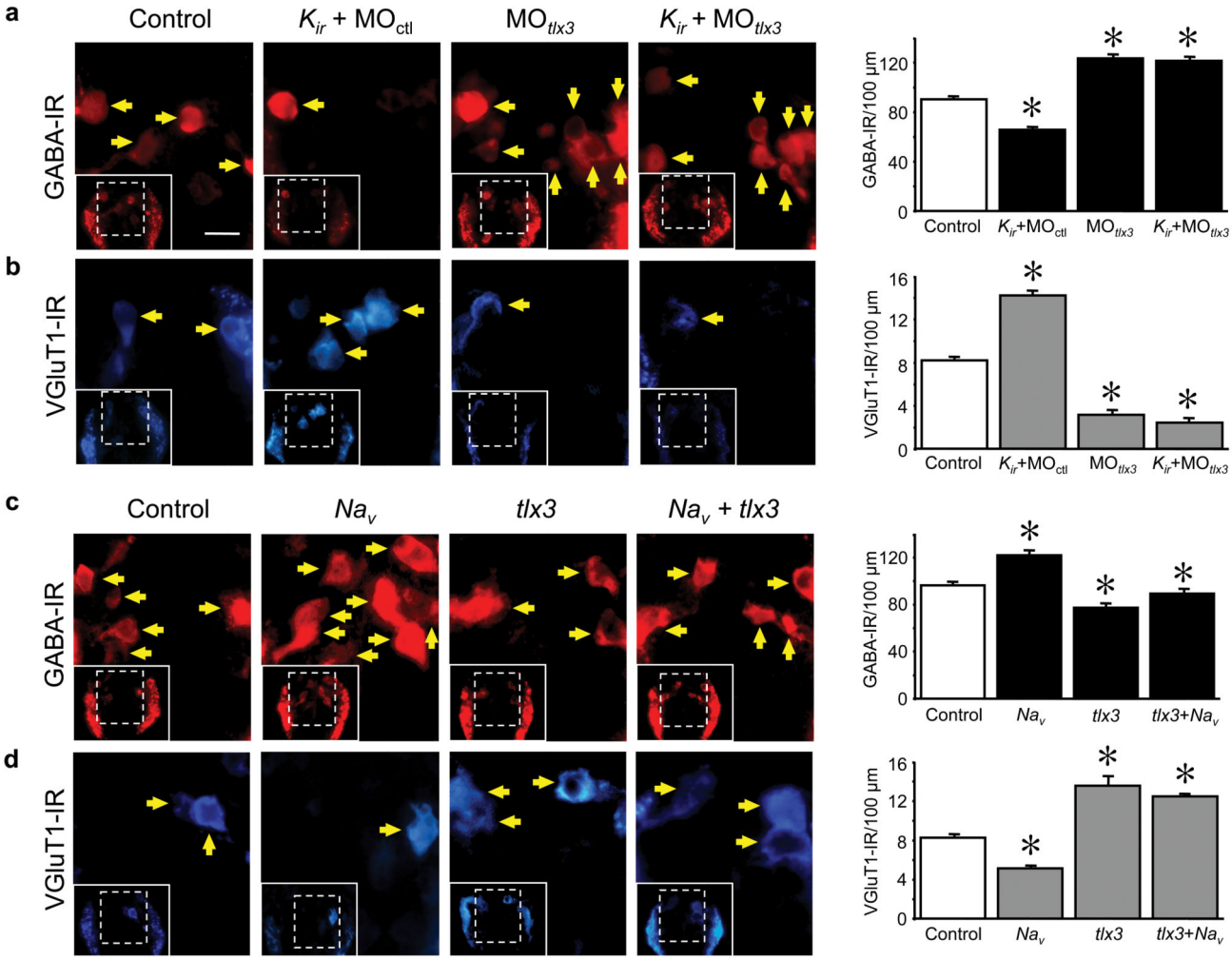

Figure 1. Activity-dependent specification of GABA and glutamate in embryonic spinal neurons requires *tlx3*

GABA and vesicular glutamate transporter immunoreactivities (GABA-IR, VGluT1-IR) for stage 41 larvae are shown at left and quantified per 100 µm of spinal cord at right. Panels show central regions of the spinal cord (dashed outlines in insets) enlarged to make cell bodies clear. Insets show the entire spinal cord, including cell bodies and lateral axon tracts. Cells included in the counts are marked with arrows; other profiles were counted in adjacent sections. **a**, GABA specification is suppressed by overexpression of potassium channels (K_{ir}) together with a control morpholino (MO_{ctl}) and enhanced by reducing expression of *tlx3* by splice-blocking morpholino injection (MO_{tlx3}). Combining K_{ir} overexpression and MO_{tlx3} injection (K_{ir} + MO_{tlx3}) yields the MO_{tlx3} phenotype. **b**, glutamate specification is enhanced by K_{ir} overexpression and reduced by MO_{tlx3} injection. Combining K_{ir} overexpression and MO_{tlx3} injection produces the MO_{tlx3} phenotype. **c**, GABA specification is enhanced by overexpression of sodium channels (Na_v) and suppressed by overexpression of *tlx3*. Combining the two perturbations generates the *tlx*3 overexpression phenotype. **d**, glutamate specification is suppressed by overexpression of sodium channels and enhanced by overexpression of *tlx3*. Combining the two perturbations yields the *tlx*3 overexpression

phenotype. Scale bar is 100 μm for insets and 25 μm for magnified panels. a–d, data are mean±SEM; n≥7 larvae; *, P<0.05.

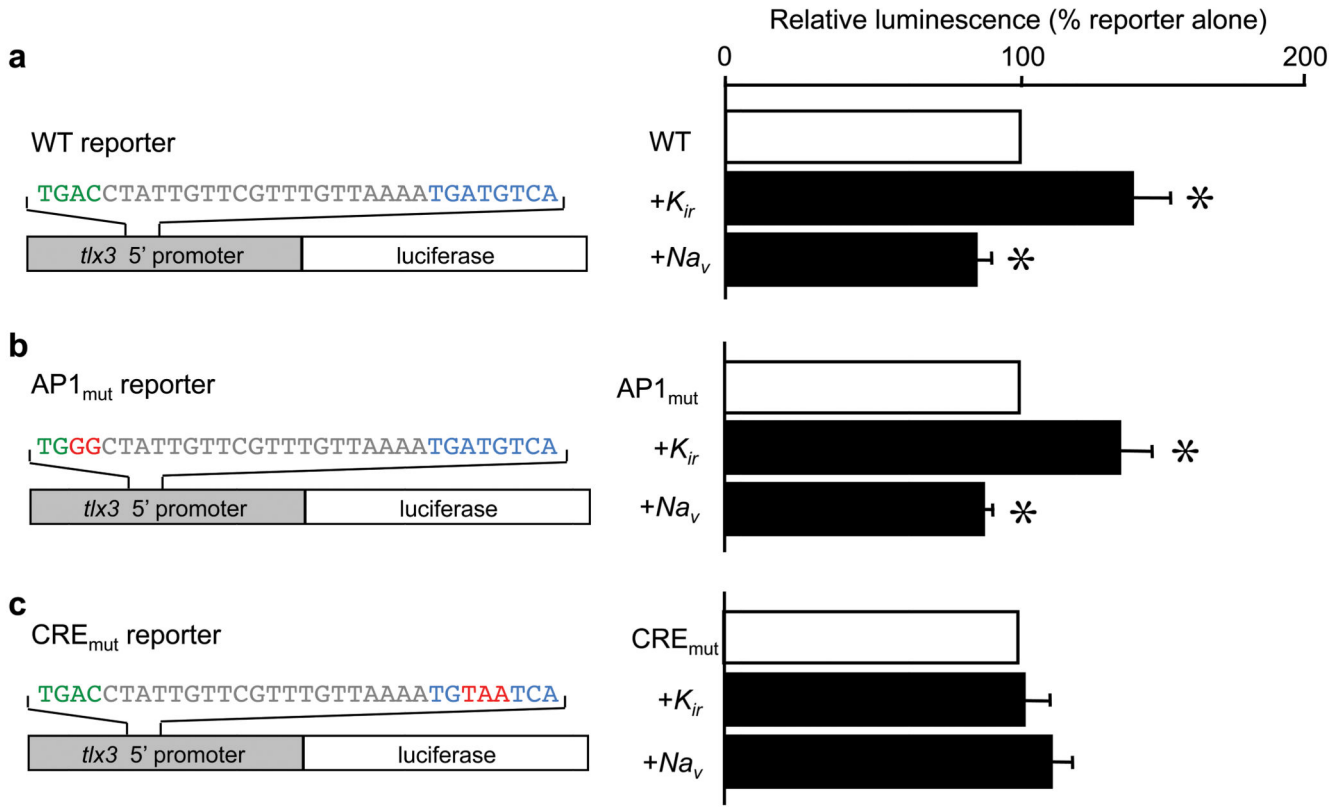

Figure 2. A variant CRE binding site is required for activity-dependent transcriptional regulation

a, a control construct in which 384 bp of the *tlx3* promoter drives firefly luciferase reports an increase in relative luminescence (RL) when coinjected with K_{ir} and a decrease in RL when coinjected with Na_v. **b**, mutating the AP1 site (red) has no effect on luciferase expression. **c**, mutating the CRE site (red) eliminates the activity-dependence of luciferase expression. b–d, data are mean±SEM; n≥3 clutches; *, P<0.05.

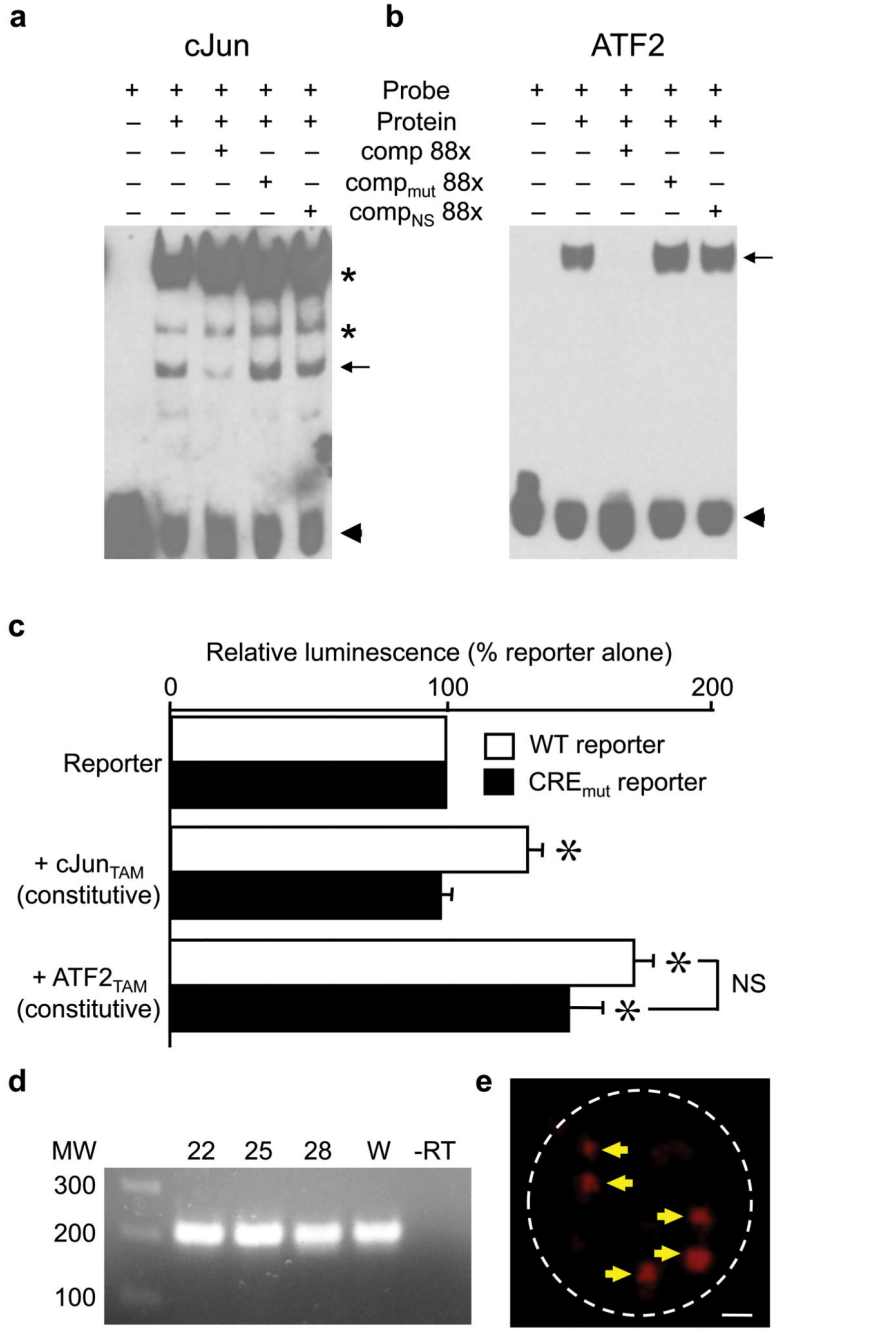

Figure 3. cJun interacts with the CRE site

a,b, electrophoretic mobility shift assays show a shift in CRE probe migration in response to cJun (**a**) and ATF2 (**b**) that is eliminated by competition with excess unlabeled probe (comp), but not an unlabeled probe with a mutant CRE (comp$_{mut}$) or a non-specific probe (comp$_{NS}$). Specific bands are indicated by arrows and non-specific bands by asterisks; free probe is indicated by arrowheads. **c**, constitutively overexpressing a dominant negative cJun or ATF2 construct lacking the transactivation domain (cJun$_{TAM}$, ATF2$_{TAM}$) increases the activity of the wildtype *tlx3* luciferase reporter, but only the increase caused by cJun$_{TAM}$

requires an intact CRE. **d**, RT-PCR performed on isolated spinal cords shows *cJun* transcripts are expressed at stages 22, 25, and 28. W: stage 28 whole embryos as positive control, -RT: same as W, without reverse transcriptase in the reaction. **e**, cJun-IR in the spinal cord of a stage 25 embryo. Scale bar is 25 μm. c, data are mean±SEM for n≥3 clutches; *, P<0.05; NS, not significant.

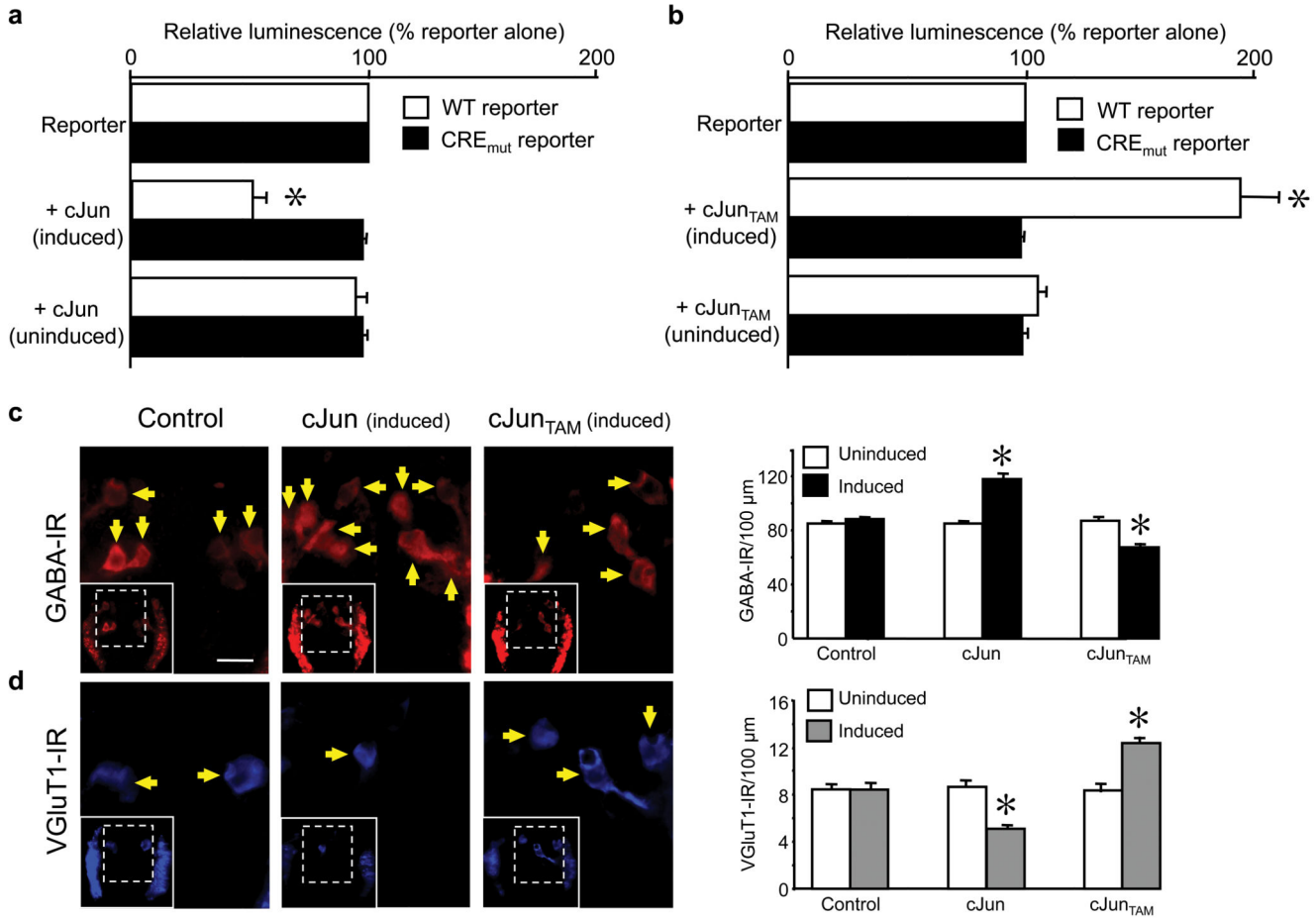

Figure 4. cJun regulates transcription and specification of GABA and glutamate

a,b, inducing overexpressed wildtype cJun at stage 24 causes a reduction in activity of the wildtype *tlx3* luciferase reporter while inducing the dominant negative cJun produces the opposite result. Mutating the CRE site abolishes these effects. **c,d**, inducing overexpressed wildtype cJun at stage 24 causes an increase in the number of GABA-IR neurons and a decrease in the number of VGlutT1-IR neurons, while inducing the overexpressed dominant negative cJun results in a decreased incidence of GABA-IR neurons and an increase in the number of VGluT1-IR neurons. Overexpression of either construct without induction has no effect. Immunostaining for GABA-IR and VGluT1-IR performed on stage 41 larvae and formatted as in Figure 1. Scale bar is 100 µm for insets and 25 µm for magnified panels. Data are mean±SEM; a,b, n≥3 clutches; c,d, n≥15 larvae; *, P<0.05.

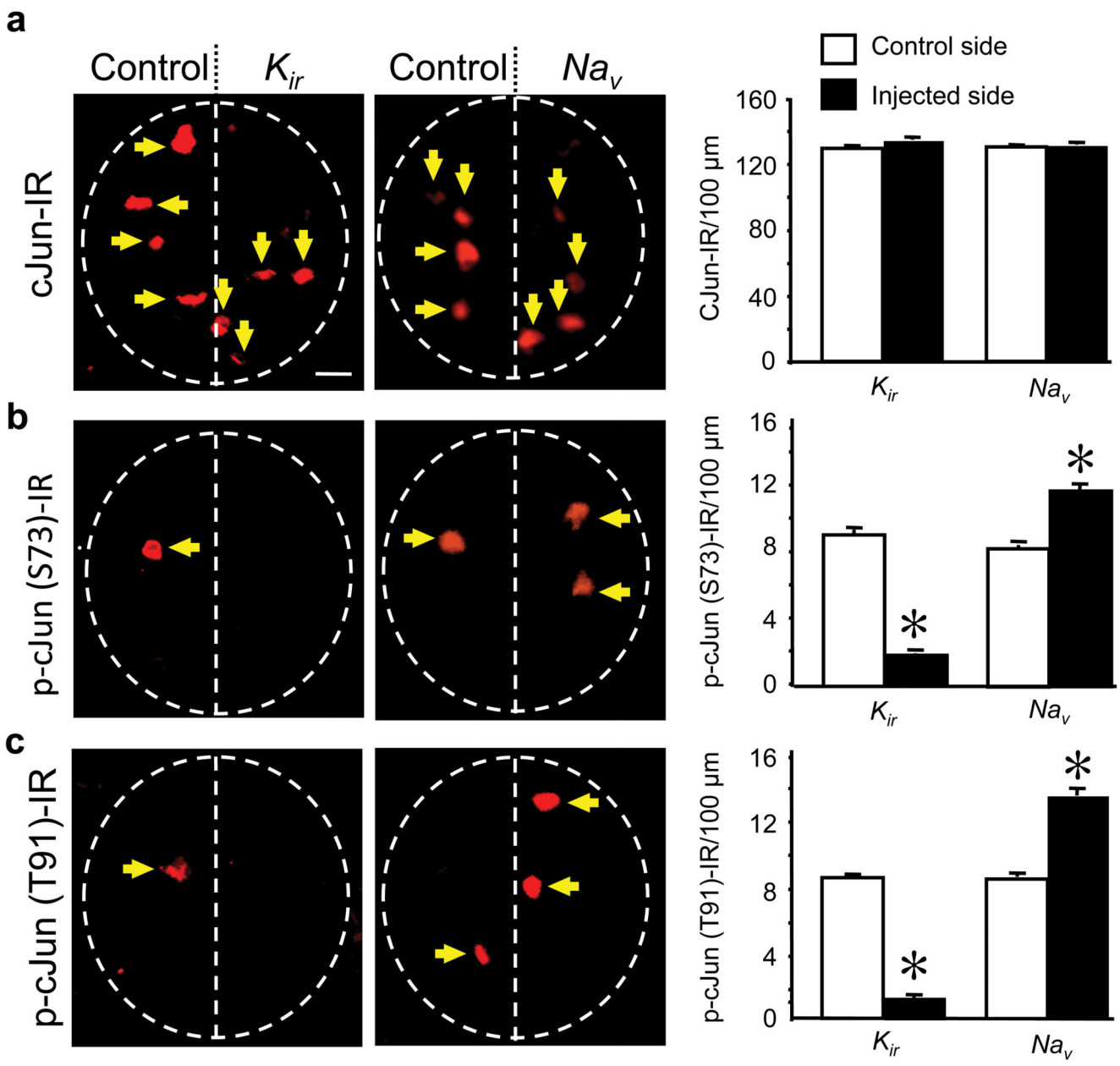

Figure 5. Ca spike activity regulates phosphorylation of cJun

a, the number of cells expressing cJun is not changed by Ca activity manipulations. **b,c**, Ca spike suppression causes a decrease in the number of cells expressing cJun phosphorylated at residues S73 [p-cJun (S73)-IR) and T91 [p-cJun (T91)-IR], while Ca spike enhancement causes an increase in the number of cells expressing S73 and T91 phosphorylated cJun. a–c, K_{ir} or Na_v was injected into one cell of two-cell embryos to suppress or enhance Ca spike activity, respectively. Immunostaining performed on stage 28 embryos. Scale bar is 25 µm. Data are mean±SEM; n≥15 embryos; *, P<0.05.

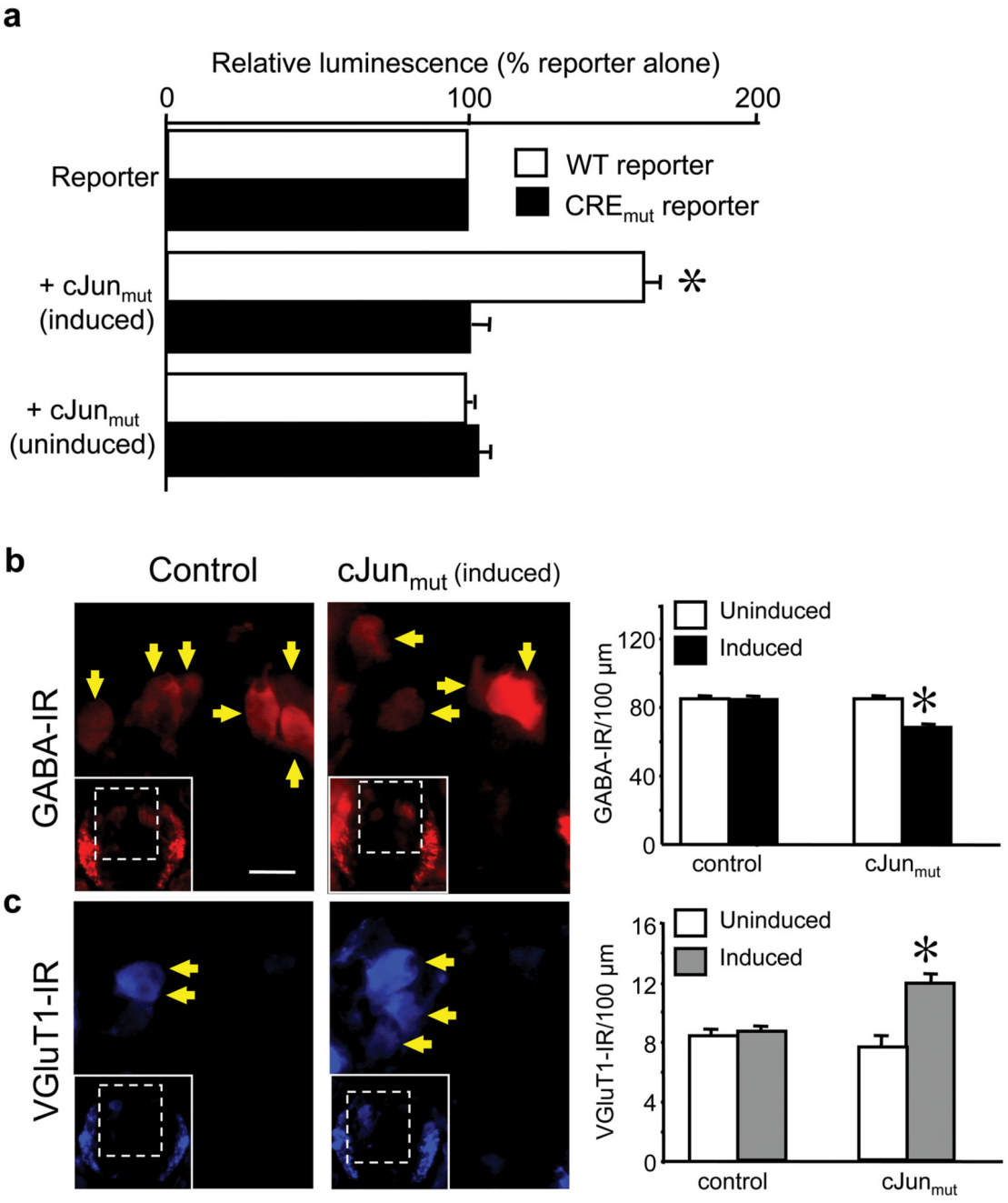

Figure 6. cJun signaling integrates genetic and activity-dependent neurotransmitter specification

a, inducing an overexpressed phosphorylation mutant of cJun ($cJun_{mut}$; S63A, S73A, T91A and T93A) at stage 24 causes an increase in the activity of the wildtype *tlx3* luciferase reporter. Mutating the CRE site abolishes this effect. **b, c**, inducing the overexpressed phosphorylation mutant of cJun at stage 24 causes a decrease in the number of GABA-IR neurons and an increase in the number of VGlutT1-IR neurons. Overexpression without induction has no effect. Immunostaining for GABA-IR and VGluT1-IR performed on stage

41 larvae and formatted as in Figure 1. Scale bar is 100 μm for insets and 25 μm for magnified panels. Data are mean±SEM; a, n≥3 clutches; b,c, n≥15 larvae; *, P<0.05.