

The alternative protein isoform NK2B, encoded by the *vnd/NK-2* proneural gene, directly activates transcription and is expressed following the start of cells differentiation

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ABSTRACT

NK-2 is a homeodomain protein essential for the development of the central nervous system in the *Drosophila* embryo. Here, we show that the *vnd/NK-2* gene encodes an additional protein isoform (NK-2B) that differs from the known one (NK-2A) in its N-terminal domain. While NK-2A is a transcription repressor, NK-2B directly activates transcription from promoters containing NK-2 binding sites, with its N-terminal domain possessing a strong transcription activation potency. The transcription of NK-2B starts at the onset of metamorphosis. Its expression is observed in precursors of differentiating photoreceptors and in photoreceptors of the adult eye. Both NK-2B and NK-2A are expressed in the lamina. However, the expression of NK-2A is mostly associated with the undifferentiated state of nervous cells.

INTRODUCTION

The nervous system of mammals or arthropods consists of thousands or hundreds of different neuronal cell types. Homeodomain (HD)-containing transcription factors play an important role in the specification of neuronal cell identities. Among them, the NK-2 family of homeo-protein transcription factors is essential for these processes. Mammalian Nkx2-2 is required for the establishment of the dopaminergic progenitor domain in the ventral midbrain, and serotonergic neurons, in the brain stem and ventral spinal cord (1,2). It has been identified as a key regulator of oligodendrocyte differentiation (3–5).

The *Nkx2.1*, the vertebrate homolog of *Drosophila Vnd/NK-2*, is essential for creating the diversity of neuronal subtypes in the brain (6).

Drosophila vnd/NK-2, initially identified as a gene encoding a HD protein (7), functions as a proneural gene that is essential for the development of a part of the embryonic central nervous system (CNS). Its expression starts early in embryogenesis, initiating neural development in the ventral neuroectoderm. At later stages, *vnd/NK-2* expression continues in part of ventrolateral neuroblasts segregated from the neuroectodermal cell layer. The mutation of *vnd/NK-2* is lethal (8). The expression of NK-2 markedly decreases in the cell progeny of differentiating neuroblasts; however, it was observed in motoneurons, interneurons and glial cells of the embryonic CNS (7,9–11).

Members of the NK-2 family type I share three conserved domains: the HD, tinman (TN) domain and NK-2-specific domain (NK2-SD) unique for the NK-2 class of proteins (7,12). The canonical NK-2 binding site, having the highest affinity for NK-2 HD, was determined (13,14). The sites of NK-2 protein binding, slightly varying in nucleotide sequences, were found in the regulatory regions of different *Drosophila* genes, including NK-2 itself (15). The TN domain located at the N-terminal part of NK-2 proteins is involved in protein–protein interactions. The function of NK2-SD is unknown.

Some members of the NK-2 family of transcription factors act as repressors, by themselves or in cooperation with Groucho, while others are transcriptional activators. The vertebrate Nkx2.1, the homolog of *Drosophila* NK-2, was shown to be a transcription activator. The potential transcription activation region was mapped in the

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Drosophila NK-2 protein. This region fused to NK-2 HD activates transcription of the reporter gene placed under the promoter bearing the canonical binding sites for NK-2 (16,17). For this reason, it can be expected that the whole NK-2 is involved in transcription activation. However, *Drosophila* NK-2 was shown to be a direct transcription repressor. It acts directly on target genes by binding to their DNA and repressing transcription in association with the Groucho co-repressor (17–19). The NK-2 protein directly represses expression of the *ind* gene, which contains three NK-2 binding sites in its regulatory region (20). The repression domain of NK-2 was mapped at the N terminus of the protein (16).

Here, we show that two alternative variants of the NK-2 protein (NK-2A and NK-2B) exist in *Drosophila*. While NK-2A is a transcription repressor, as shown previously, NK-2B has proved to be a transcription activator. This is explained by the substitution of an activator domain for the repressor domain encoded by the first exon. While the expression of NK-2A takes place mainly during embryonic development, the expression of NK-2B is associated with the onset of metamorphosis, being observed in photoreceptor cells of eye–antennal imaginal disks and in photoreceptors of the adult eye.

MATERIALS AND METHODS

Plasmid construction and cells transfection

The reporter plasmids pUC/5NK2hsp43-gal was described previously (16). To express the full-length NK-2A and NK-2B isoforms, the corresponding cDNAs were cloned in expression vector pIB (pIB/V5-His) (Invitrogen). Construct (–40)NK-2B cloned in pIB/V5-His consisted of the NK-2B sequence without the first exon; construct 40 HD contained the first exon of NK-2B gene fused with amino acids 538–620 of the NK-2 HD; and construct HD contained the HD alone. To construct the –0.75 NK-2B, –1.9 NK-2B and –3.4 NK-2B plasmids, different fragments of the NK-2B promoter region were polymerase chain reaction (PCR)-amplified using the NP6-1 plasmid containing the 14.3-kb fragment of the *vnd/NK-2* promoter region and cloned in the pUC/hs43-gal reporter plasmid. Transient transfection of *Drosophila* S2 cells and the reporter gene assay were performed as described (16).

Messenger RNA preparation, northern blotting and reverse transcriptase–PCR

RNAs from embryos at different times after fertilization, third instar larvae and adult flies were isolated using a Quick Prep Micro mRNA Purification Kit (Amersham Bioscience) according to the manufacturer's protocol. For northern blot analysis, equal amounts of RNA (0.5 µg) were resolved by electrophoresis in 1% denatured agarose gel and hybridized with ³²P-labeled antisense *vnd/NK-2* messenger RNA (mRNA) used as a probe. The first cDNA strand was synthesized with a RevertAid First Strand cDNA Synthesis Kit (Fermentas). The products of PCR were resolved in 1.2% agarose gel.

Chromatin immunoprecipitation and quantitative PCR analysis

Chromatin immunoprecipitation (ChIP) was performed according to the published procedures (21,22). DNA was sheared to a size of about 300 bp. After ChIP, the recovered DNA was analyzed by quantitative (q)PCR with MiniOpticon (BioRad). Sequences of the primers are given in Supplementary Data. Each point was measured in at least five experiments, and the mean value was calculated. For baseline (control) measurements, ChIP with preimmune IgG was used.

In situ hybridization

The 1B exon (120-bp PCR product) or 1A exon (180-bp fragment) was cloned into BlueScript (Roche) and used for the synthesis of antisense RNA according to manufacturer's instructions. Hybridization with biotin- or digoxigenin-labeled RNA probes was performed according to the Berkeley *Drosophila* Genome Project protocol, with some modifications.

Antibodies and immunohistochemistry

Polyclonal antibodies against NK-2A were raised in rabbits. Second and third instar larvae and adult flies were fixed in a 3.8% formaldehyde solution in phosphate-buffered saline (PBS) to dissect imaginal disks and the brain. To reveal NK-2 proteins, preparations were treated with rabbit anti-NK-2 antibody (1:1000) overnight at 4°C and then with goat Cy3 anti-rabbit antibody (Jackson ImmunoResearch) diluted 1:500. Staining for the 3HA-Ig marker involved treatment with mouse anti-3HA antibody (Sigma) (diluted 1:1000) and with goat Cy2 anti-mouse antibody (Jackson ImmunoResearch) diluted 1:800. After staining, tissue preparations were preserved in the Vectorshield medium (Vector Laboratories) and examined under a fluorescent or confocal microscope.

RESULTS

The *vnd/Nk-2* gene encodes an additional transcript, which is expressed from an alternative promoter at postembryonic stages of development and differs in the first exon

Since the NK-2 transcription repressor contains the potential transcription activation region, we were interested if its alternative forms that are capable of activating transcription exist in *Drosophila*. To reveal the alternative transcripts of *vnd/NK-2*, we analyzed its possible expression at different stages of *Drosophila* development. Northern blot analysis revealed two transcripts, 3.4 and 2.8 kb in size, hereinafter referred to as NK-2A and NK-2B, respectively (Figure 1A). The expression of the NK-2A transcript starts in 3-h embryos, correlating with the onset of zygotic transcription, and its level increases at subsequent stages of embryonic development. Interestingly, this transcript was also detected in larvae and adults, but its level was dramatically reduced at this stages. Conversely, NK-2B mRNA was absent in embryos but appeared beginning from larval stages. In the larvae and adults, its level was higher than that of the NK-2A

transcript. The length of the NK-2A transcript coincided with that determined for *vnd*/NK-2 mRNA, which is expressed in embryos, with the nature of NK-2B mRNA remaining unknown.

Northern blot hybridization with probes corresponding to different regions of *vnd*/NK-2 provided evidence that NK-2B mRNA lacked the first exon found in NK-2A mRNA. On the other hand, reverse transcriptase (RT)-PCR analysis showed that the NK-2B transcript contained specific sequences located 8.2 kb upstream of the known NK-2 transcription start site. Next, the expression pattern of NK-2A and NK-2B mRNA in the adult fly was studied by RT-PCR with primers specific for each transcript. The NK-2B mRNA was detected in both the head and body, while the NK-2A mRNA was detected only in the head of the adult fly (Figure 1C). Full-length cDNAs containing 5' and 3' untranslated regions of both transcripts were obtained by RT-PCR on mRNAs isolated from the heads or bodies of adult flies (see Supplementary Data). Sequencing confirmed that the NK-2B transcript lacks the first exon (1A) but contains an alternative exon 118 bp in size (exon 1B), which is fused with the second exon of NK-2 (Figure 1B). Thus, this transcript can potentially encode a novel protein differing from the known NK-2 isoform in the N terminus.

The TATA box-containing promoter for the NK-2B transcript was revealed 8.2 kb upstream of the known NK-2 transcription start site, whereas the transcription of NK-2A mRNA is known to be driven by the TATA-less promoter (17). The region upstream of NK-2B promoter contains the predicted sites for binding several transcription factors participating in *Drosophila* development. In particular, nine binding sites were found for proteins encoded by the *Broad-Complex* (*BR-C*) gene,

which is essential for gene activation during metamorphosis (23).

The NK-2B transcript encodes the protein detected in tissues of *Drosophila melanogaster*

We resorted to rabbit polyclonal antibodies against the recombinant NK-2A which were expected to recognize both NK-2 isoforms. These antibodies proved to be highly specific in immunostaining experiments (Supplementary Figure S1A), revealing the characteristic pattern of NK-2 distribution in the *Drosophila* embryo (24).

The presence of the NK-2B isoform in the adult fly body was assessed by western-blot analysis (Figure 2A). The antibodies detected only the protein band of the molecular weight corresponding to that calculated for NK-2B. With respect to electrophoretic mobility, this band coincided with recombinant NK-2B expressed in S2 cells and differed from NK-2A. A noteworthy fact is that NK-2B migrated as a set of bands with close electrophoretic mobilities, which apparently reflected its posttranslational modifications. A similar pattern of closely migrating bands was observed for NK-2A (Figure 2A). Thus, the NK-2B mRNA encodes the alternative protein isoform, and, as shown by NK-2 transcription data, its expression starts at the larval stage of development (Figure 1A).

The two NK-2 isoforms differed in the N-terminal regions encoded by the first exons (Supplementary Figure S1B), while other domains, including the potential activation region between TN and HD, were the same in both proteins (Figure 2B). NK-2B has acquired a sequence of 40 N-terminal amino acids (mostly hydrophobic) that has replaced the repression domain of NK-2A.

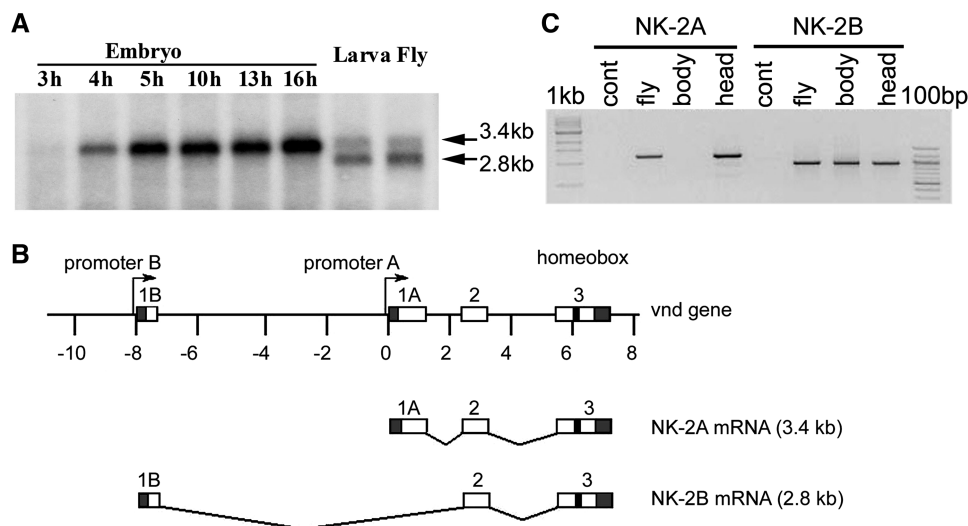


Figure 1. NK-2 encodes two mRNAs transcribed from alternative promoters. (A) Northern blot hybridization demonstrates the expression of two NK-2 transcripts in embryos (3, 4, 5, 10, 13 and 16 h after fertilization), third instar larvae and adult flies. On each lane, 0.5 μ g of total mRNA was loaded, and 32 P-labeled *vnd*/NK-2 antisense mRNA was used as a probe. (B) The exon/intron structure of the *D. melanogaster vnd*/NK-2 gene and its alternative transcripts. Arrows indicate alternative transcription start sites. Boxes show exons with coding regions (white), untranslated regions (gray) and the homeobox region (black). (C) Expression of NK-2 isoforms in *D. melanogaster* as assessed by RT-PCR with primers specific for each isoform. Sample RNAs were from whole flies, heads and bodies (indicated above the panel); in the control (cont), RT-PCR was performed without RNA; 1 kb and 100 bp are corresponding markers.

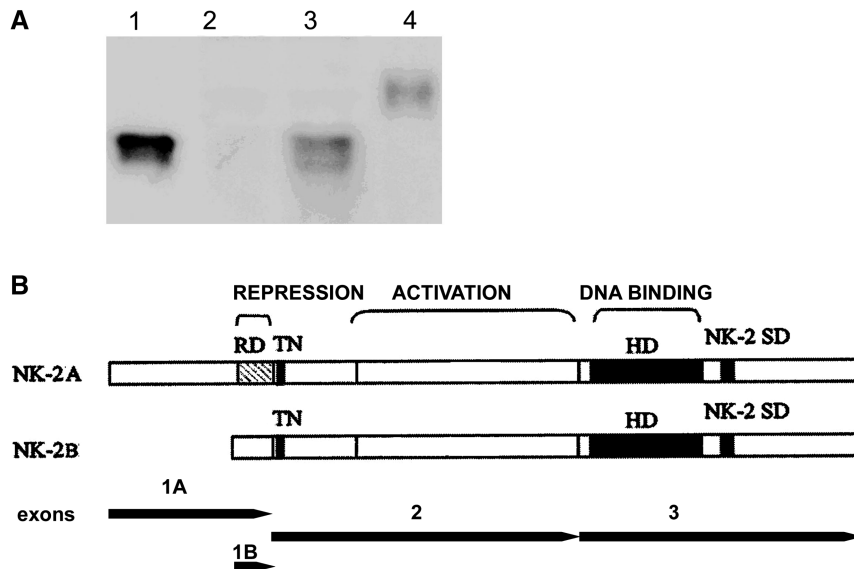


Figure 2. Expression and structure of the NK-2B protein. (A) The results of western blot analysis show that the NK-2B protein is expressed in adult fly tissues. Total protein extracts from S2 cells transfected with the expression construct for NK-2B (lane 1), untransfected S2 cells (lane 2), adult fly bodies (lane 3) and S2 cells transfected with the expression construct for NK-2A (lane 4) were resolved in 6% sodium dodecyl sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and stained with rabbit anti-NK-2A antibody and then with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody. Note that the set of NK-2B-positive bands in the extracts from fly tissues and transfected S2 cells is the same, indicating that S2 cells provide for correct NK-2 folding and, therefore, for its proper functioning in DNA binding and transcription regulation. (B) Scheme of the domain structure of NK-2A and NK-2B proteins. Conserved NK-2 domains TN, HD and NK-2 specific domain (NK-2 SD) are shown in black, and their functions are indicated. Both proteins contain an additional activation region between TN and HD. The repression domain of NK-2A is located immediately before the TN domain. The NK-2B isoform lacks N-terminal sequences, including the repression domain, which is replaced by the novel domain located at the same position.

The N-terminal region encoded by exon 1A was predicted to be a disordered low-complexity region [Eukaryotic Linear Motif (ELM) database], while that encoded by exon 1B was predicted to have a globular structure. In addition, the NK-2B N-terminal region has potential sites for interaction with several transcription factors, which may contribute to NK-2B functioning. These differences may account for different functions of NK-2A and NK-2B proteins in transcription regulation.

In contrast to NK-2A, which represses transcription, NK-2B is a transcription activator

The next step was to determine whether NK-2B, which lacks the N-terminal repression domain, can function as a transcription activator. To this end, we constructed a plasmid for NK-2B expression in *Drosophila* S2 cells (below, referred to as the NK-2B plasmid) by cloning the cDNA of the *NK-2B* gene into the pIB/V5-His plasmid (Figure 3A). The influence of NK-2B on transcription level was estimated using the pUC/5NK-2hsp40-gal plasmid with the β -galactosidase reporter gene placed under control of the 5-NK-2 promoter, which contained a TATA box from *hsp70* deleted at -43 (*hs43*) and five canonical NK-2 binding sites (16) (Figure 3B). The S2 cells were co-transfected with both plasmids. In control experiments, the NK-2B plasmid was replaced either by pIB/V5-His plasmid expressing full-length NK-2A or by an empty pIB/V5-His vector.

It is noteworthy that, according to the results of immunostaining experiments (data not shown), the

concentrations of recombinant NK-2A or NK-2B expressed in the transfected cells were even slightly lower than in *Drosophila* tissues, which was probably due to a certain molecular mechanism regulating the NK-2 level in the cells. Thus, our experiments were performed at NK-2 isoform concentrations similar to those existing *in vivo*.

In S2 cells transfected with NK-2B, the transcription level of the reporter gene was ~ 12 times higher than in cells transfected with the pIB vector (Figure 3C). In agreement with previous data (16), co-transfection with NK-2A resulted in a 5-fold decrease in reporter gene transcription. ChIP experiments confirmed that both isoforms bound the promoter of the reporter construct at approximately the same level and, therefore, the influence of NK-2 isoforms on reporter gene expression was not due to their different binding capacities (Figure 3D).

Thus, NK-2A acted as a transcription repressor, but NK-2B proved to activate transcription from the promoter containing the canonical NK-2 binding sites. This could be explained in two ways: either the deletion of the NK-2A N-terminal region (exon 1A) allowed the functioning of the NK-2 activation domain or the novel N-terminal peptide present in NK-2B activated transcription. To evaluate the role of this peptide (1B), we compared transcription levels induced by whole-length NK-2B and by NK-2B lacking exon 1B (Figure 3A). The results showed that the 1B exon deletion resulted in a dramatic drop in the transcription level (Figure 3E).

We then estimated if exon 1B alone could activate transcription. To this end, we determined the transcription level of the reporter gene induced by exon 1B fused to

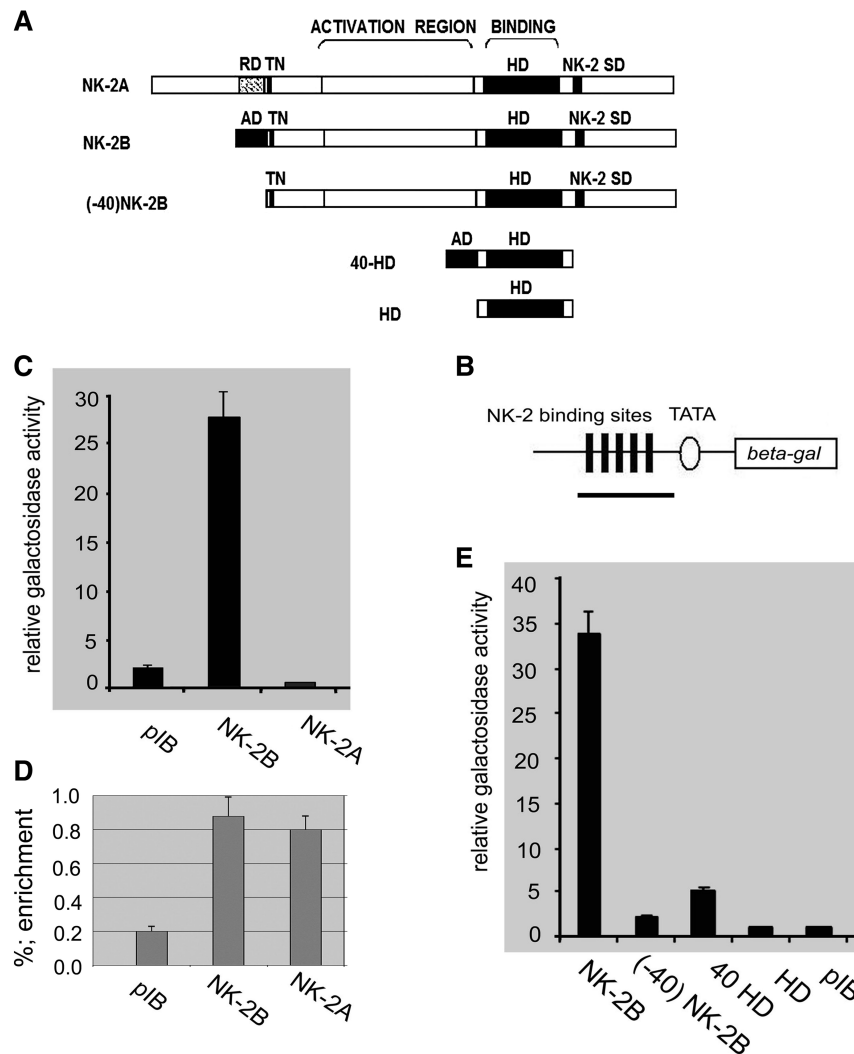


Figure 3. The NK-2B isoform activates transcription. (A) Schemes of NK-2-expressing constructs used in experiments. (B) Scheme of the 5-NK-2 reporter construct: black bars are canonical NK-2 binding sites TCAAGTG, and white ellipse is the TATA box. The DNA fragment amplified in ChIP experiments is underlined. (C) Full-length NK-2B activates transcription of the 5-NK-2-TATA promoter. S2 cells were co-transfected with the NK-2A- or NK-2B-expressing construct and the reporter 5-NK-2 construct; in the control, the former construct was replaced by an empty pIB/V5-His vector. The vertical axis shows the expression level of the reporter β -galactosidase gene. (D) The distribution of NK-2A and NK-2B on the 5-NK-2 binding sites assayed by ChIP and quantitative PCR analysis. The position of the fragment used in ChIP experiment is indicated in Figure 3B. Cells were co-transfected with construct expressing either NK-2A or NK-2B or an empty vector (control) and with the 5-NK-2 reporter construct. The results are shown as the percentage of input, with the baseline level (ChIP with preimmune IgG) varying from 0.02 to 0.05% at different points. Each point was measured in at least five experiments, and the mean value was calculated. The amounts of NK-2A, NK-2B and empty vector expressed in cells were equal (as tested at both DNA and protein levels), and the amount of the reporter construct was the same in different experiments. (E) Exon 1B activates transcription. Experimental conditions were as in Figure 3C.

the NK-2 HD (Figure 3A). The results showed that transcription in this case was increased significantly relative to the control (induction by the HD without exon 1B) (Figure 3E). Thus, exon 1B alone was capable of activating transcription from the 5-NK-2 promoter. Taking into account that the NK-2 HD slightly repressed transcription in this system (16), the activation potency of the 1B domain could be estimated at $\sim 60\%$ relative to that of full-length NK-2B.

NK-2B activates its own promoter

The next question was regarding the involvement of NK-2A or NK-2B in regulation of the NK-2B promoter.

An analysis of the region upstream of the NK-2B promoter revealed the presence of NK-2 binding sites: one site immediately upstream of the NK-2B TATA box, four sites in proximity to the promoter (-1.9 kb) and six more sites in the region between -1.9 and -3.8 kb (Figure 4A). The plasmids bearing the β -galactosidase reporter gene driven by the *hs43* promoter under control of the NK-2B regulatory region sequentially truncated from the 5' end (Figure 4A) were constructed to test the influence of NK-2B and NK-2A on their transcription.

The results showed that NK-2B strongly activated transcription from its own promoter (Figure 4B). The highest transcription level was observed in the -1.9 -kb construct

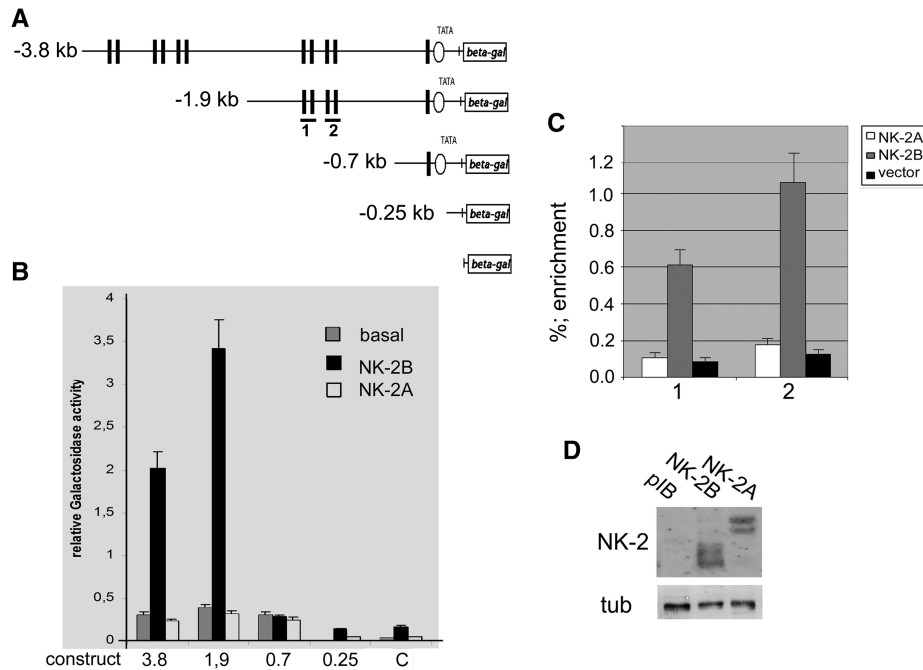


Figure 4. Effects of NK-2B and NK-2A on activation of transcription from the NK-2B promoter. (A) Schemes of constructs containing different fragments of the NK-2B regulatory region (cloned upstream of the *hs43* promoter) and the reporter gene that were used in transfection experiments. Black bars are canonical NK-2-binding sites. (B) Effects of NK-2B and NK-2A on transcription of the above constructs. The level of their transcription in S2 cells is taken as the baseline. (C) The distribution of NK-2A and NK-2B on the NK-2 binding sites of the regulatory region of NK-2B gene, assayed by ChIP. Positions of fragments used in ChIP experiments are indicated in Figure 4A (construct 1.9). Other experimental conditions were as in Figure 3D. (D) The results of western blot analysis of total protein extracts from S2 cells transfected with pIB vector or with constructs expressing NK-2B or NK-2A proteins. Staining with rabbit anti-NK-2A antibody, tubulin (tub) was used as a loading control.

that contained five NK-2 binding sites. Four of them, located more distantly from the start of transcription, appear to be the most important for maintaining a high transcription level, since NK-2B had no effect on the transcription of the reporter gene after their deletion (in the -0.7 -kb construct). The six distal sites showed no significant contribution to transcription activation (compare the results for the -3.8 - and -1.9 -kb constructs). After the total deletion of genomic region containing NK-2 binding sites, the transcription of the reporter gene decreased to the baseline level.

It was expected that NK-2A would inhibit transcription from the NK-2B promoter as well as it inhibited transcription from the 5-NK-2 promoter (Figure 3). In fact, NK-2A had almost no influence on transcription from the NK-2B promoter (Figure 4B).

To find an explanation to this fact, we used ChIP to analyze for the presence of NK-2A and NK-2B at four NK-2 binding sites of NK-2B promoter that, according to our results, had the strongest effect on the NK-2B expression level (Figure 4B). Cells were transfected with the construct expressing either NK-2A or NK-2B, and the presence of NK-2 isoforms was first studied on endogenous NK-2B promoter. A high level of NK-2B was detected at all NK-2 binding sites (Figure 4C). The binding of NK-2A to these sites was at the level of control (transfection with an empty vector). The difference in binding capacities of two isoforms with the endogenous promoter indicated that chromatin plays a role in their interaction with NK-2 binding sites.

Next, we tested if NK-2B and NK-2A bind to the same sites in the -1.9 -kb construct (Supplementary Figure S2). The experiments were performed on cells co-transfected with this construct. In agreement with the previous experiment, its presence had no effect on the distribution of either isoform: NK-2A did not interact with NK-2 binding sites, while NK-2B was present there. The efficiency of transformation with reporter plasmids and plasmids expressing NK-2 isoforms as well as the levels of both isoforms in cells were controlled in all experiments. The levels of NK-2B and NK-2A in transfected cells proved to be almost equal (Figure 4D), suggesting that their different binding to NK-2B regulatory region was not due to the difference in their concentration.

Thus, our experiments showed that NK-2A failed to bind to NK-2 binding sites in the NK-2B regulatory region, which could explain its inability to repress NK-2B transcription. This result also strongly implies that NK-2A does not regulate the NK-2B promoter *in vivo*. Indeed, ChIP experiments with 12-h embryos did not reveal the presence of NK-2A on the NK-2B promoter or on promoter of the *hsp70* gene used as a negative control (Supplementary Figure S3). In the same time, NK-2A was detected on NK-2 binding sites of *Ind* gene identified before (20).

The difference in binding activity between the two isoforms may be accounted for by chromatin and DNA sequences surrounding the core binding motif. The influence of such sequences on NK-2A binding activity was shown previously (25). Moreover, the first exons of these

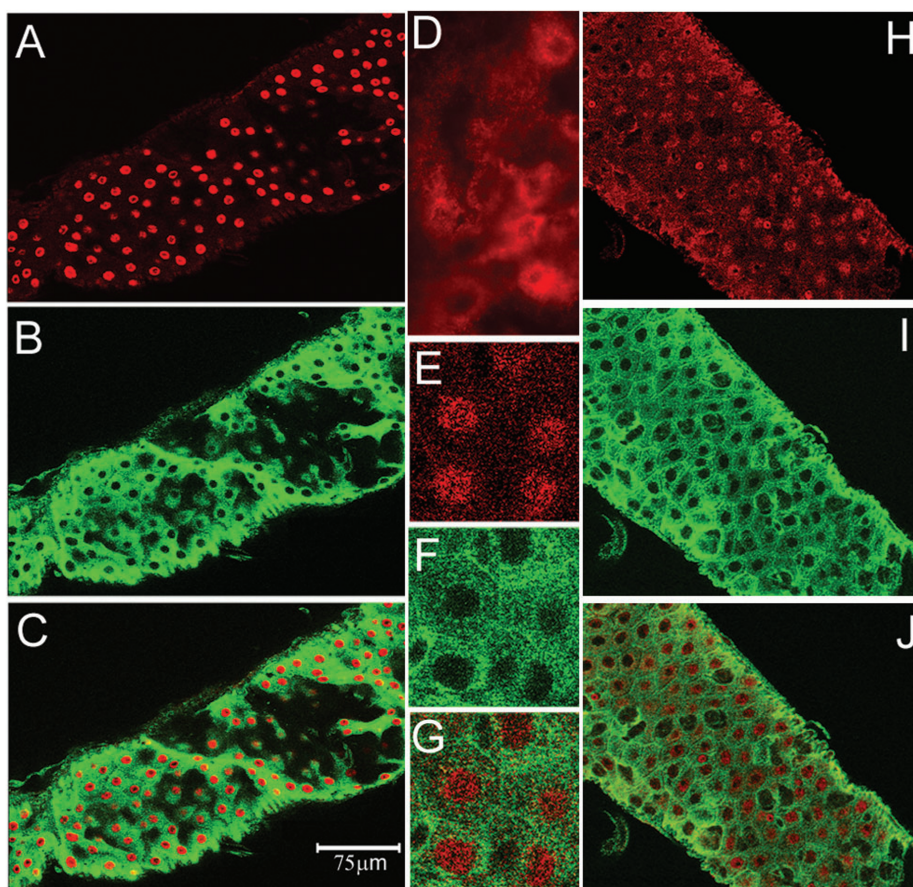


Figure 5. The expression pattern of NK-2B in the guts of (A–D) adult flies and (E–J) third instar larvae revealed by immunostaining (A–C, E–J) and *in situ* hybridization (D). Mouse anti-HA antibodies detecting NK-2B expression (green staining) and anti-NK-2 antibodies (red staining) were used; (E–G) are scaled images of (H–J) showing that, while staining with anti-HA antibodies is confined to the cytoplasm, anti-NK-2 antibodies have a nuclear staining pattern. Some cells on images H and G display weak or peripheral nuclear staining with anti-NK-2 antibodies because the image focus is in the plane of nucleoli, which occupy a large portion of the nucleus in these cells.

isoforms also appear to contribute to the above difference, since they may have an effect on their conformation and ability to interact with nucleic acids and docking proteins.

Endogenous NK-2B activates transcription in fly tissues

To verify if endogenous NK-2B is a transcription activator and to study its expression pattern, we constructed the transgenic *Drosophila* line IgHA bearing the reporter construct pCaSpeR/5NK-2-Ig-HA. The regulatory region of the construct contained five NK-2 binding sites placed upstream of the *hp43* TATA-box promoter. This construct expressed the κ light chain of Ig fused to HA-taq (see ‘Materials and Methods’ section). Thus, if NK-2B activates transcription, then cells expressing this isoform should also express HA-Ig.

According to our data, no NK-2B was expressed at the embryonic stage; therefore, embryos of the IgHA strain were used as a negative control. Indeed, while anti-NK-2 antibodies revealed the classical NK-2A expression pattern in these embryos, anti-HA antibodies failed to detect any signal, which was evidence for specificity of the transgene expression. This specificity was also confirmed by the fact that, in our subsequent experiments, we never observed

anti-HA staining in cells negative for NK-2 antibody staining.

Next, the expression of the reporter gene was studied in the adult fly body, where only NK-2B transcript was detected in previous experiments (see above). Immunostaining with anti-NK-2 antibodies revealed the expression of NK-2B only in certain regions of the gut (Figure 5A). A strong anti-HA signal was observed in the same regions, indicating a high level of transgene expression (Figure 5B). The results of *in situ* hybridization (Figure 5D) confirmed that only NK-2B was present in the gut. Thus, in line with the results obtained with S2 cells, these experiments show that NK-2B activates transcription in *Drosophila* tissues.

Anti-NK-2 antibodies proved to stain the same regions of the gut in third instar larvae. Corresponding scaled images (Figure 5E–J) clearly show the nuclear pattern of anti-NK-2 staining and the presence of anti-HA signal in the cytoplasm.

NK-2B is expressed in photoreceptor neurons since the onset of their differentiation

In third instar larvae of the IgHA line, NK-2B expression was detected in the eye domain of the eye–antennal imaginal disk (Figure 6A–H), which gives origin to

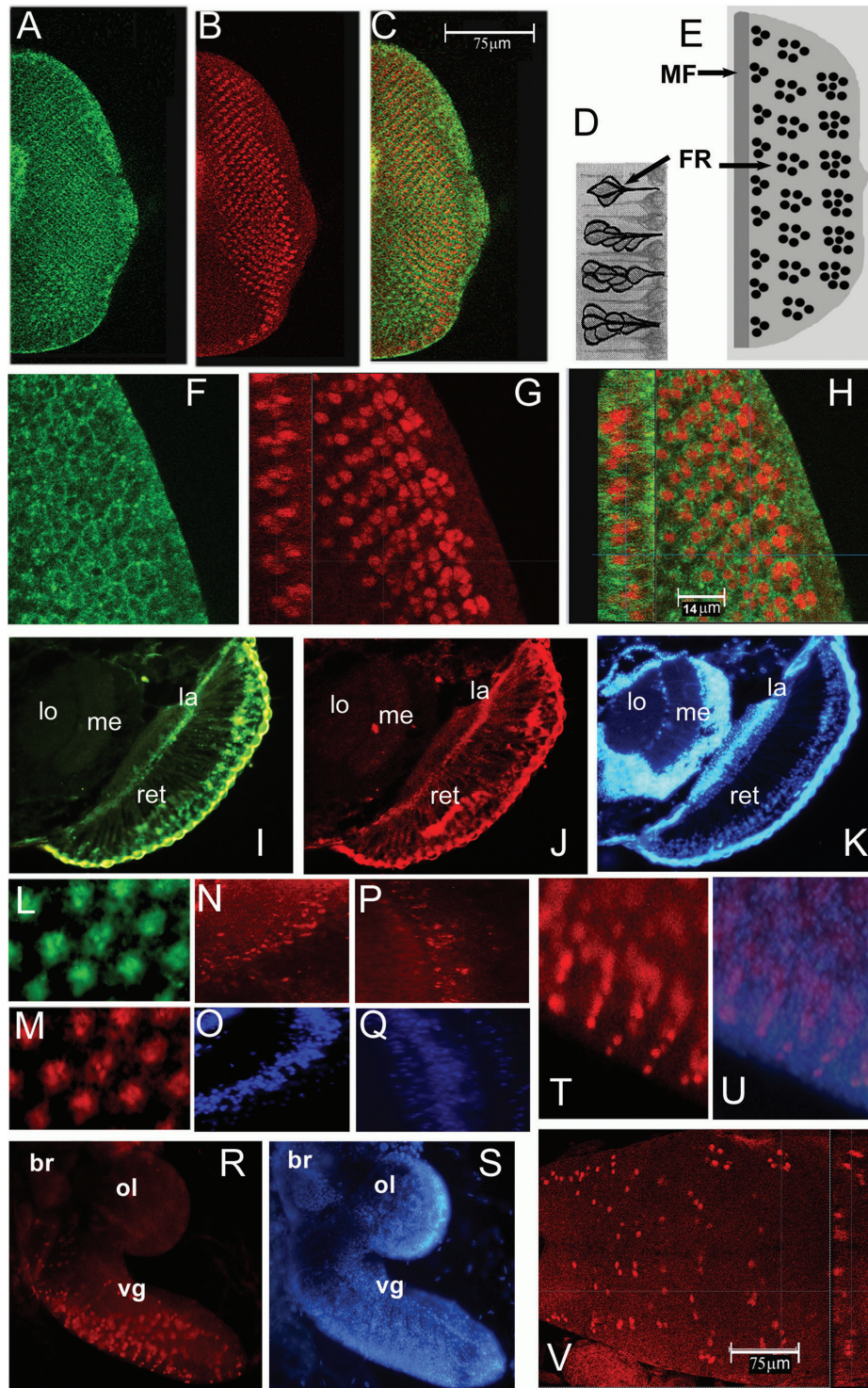


Figure 6. Expression patterns of NK-2 isoforms in the CNS of larvae and adult flies revealed by immunostaining (A–C, F–M, R–V) and *in situ* hybridization (N–Q). Immunostaining preparations were treated with anti-HA antibodies to detect NK-2B expression (green staining), anti-NK-2 antibodies detecting both isoforms (red staining) and DAPI to detect DNA (blue staining). (A–H) NK-2 expression in the eye imaginal disk. (A–C) Immunostaining of the eye imaginal disk in the region posterior to the MF. (D) Scheme of the eye imaginal disk in cross section. Differentiation of photoreceptor neurons is accompanied by migration of their nuclei to the apical side of the disk, while the nuclei of undifferentiated cells remain at the basal side. (E) Scheme of the eye imaginal disk posterior to the morphogenetic furrow (MF) with clusters of differentiating photoreceptor cell nuclei (FR). (F–H) Immunostaining of the eye imaginal disk posterior to the MF (confocal section). (I–K) Expression of NK-2 isoforms in the adult eye. (I–K) Immunostaining of the retina (re), lamina (la) and lobula (lo) of the adult eye. (L, M) Immunostaining of photoreceptor cells in the retina. (N–Q) Distribution of NK-2B mRNA (N–O) and NK-2A mRNA (P, Q) in the retina and lamina of the adult eye as revealed by *in situ* hybridization (red) and DAPI staining (blue). (R–V) NK-2A expression in the larval CNS. (R, S) The CNS stained with antibodies against NK-2 (red) and DAPI (blue); ol, optic lobe; br, brain; vg, ventral ganglion. (T, U) The scaled-up image of the part of ventral ganglion shown in (R). (V) A confocal section of the ventral ganglion stained with antibodies against NK-2.

ommatidia of the adult eye retina. Differentiation of ommatidium photoreceptor cells is initiated in the middle of the third instar by the formation of the dorsoventral morphogenetic furrow (MF) (Figure 6E). As undifferentiated cells enter the MF, they are arrested in the G1 phase of the cell cycle and cluster together to begin forming ommatidia. The resulting clusters are arranged in a hexagonal array, which accounts for the invariable honeycomb pattern of ommatidia in the adult eye. The clustered cells enter an ordered differentiation pathway leading to sequential formation of eight photoreceptors in each ommatidium (26). The nuclei of differentiating cells move toward the apical surface of the cell layer (Figure 6D). Staining with anti-HA antibodies detected the expression of NK-2B in the zone posterior to MF (Figure 6A and F). The anti-NK-2 antibody stained the same area, revealing clusters of photoreceptor cell nuclei in a regular lattice-like array (Figure 6B and G). In a horizontal section (Figure 6H), it is clearly seen that the nuclei of differentiating photoreceptors are displaced toward the apical surface of the eye disk.

The NK-2B expression was also observed in the retina of the adult eye, which is formed from the larval eye imaginal disk (Figure. 6I–K). The bodies of photoreceptors reside in the apical layer of the retina, and their axons extend toward interneurons of the lamina. In scaled images of the retina, the anti-HA and anti-NK-2 signals were clearly detected in the bodies of photoreceptors within each ommatidium (Figure 6L and M). Thus, the expression of NK-2B starts in larval photoneurons at the initial stage of their differentiation and continues in adult photoreceptors.

In addition to the retina, both antibodies stained the region of lamina but not any other regions of the eye (Figure 6I–K). *In situ* hybridization confirmed that NK-2B was expressed both in the retina and in the lamina (Figure 6N and O). A noteworthy fact is that NK-2A in the adult eye was detected in the lamina but proved to be absent in photoreceptors of the retina, as confirmed by *in situ* hybridization (Figure 6P and Q).

NK-2A is expressed in the larval CNS

The expression of the NK-2A isoform alone was observed in CNS cells of third instar larvae (Figure 6R–V). At this stage, the larval CNS starts to be replaced by the adult CNS, and neuroblasts that have previously given origin to postembryonic neurons reappear on the outer CNS surface. They start to divide in a stem cell mode to produce presumptive adult neurons, which lie deeper from the surface than neuroblasts; their nuclei are significantly smaller and form clusters beneath the latter cells. The expression of NK-2A at this stage was detected only in neuroblasts on the ventral surface of the ventral ganglion, being absent in other regions of the CNS (Figure 6R and S, vg). The expression of NK-2A proved to have a segmental pattern (Figure 6T and U). The confocal image (Figure 6V) shows clusters of NK-2A-positive nuclei inside the brain that may correspond to derivatives of neuroblasts (presumptive adult

neurons). No expression of NK-2A was detected by immunostaining in the adult fly brain.

DISCUSSION

We have described a new isoform of the *Drosophila* NK-2 protein, named NK-2B, which structurally and functionally differs from the conventional isoform (NK-2A); delimited the genomic region in which its transcription starts; demonstrated that NK-2B is a positive transcription activator; and characterized the expression patterns of both isoforms in the larva and adult fly.

While NK-2A that contains both activation and repression domains was shown to be a direct transcription repressor (16), our results provide strong evidence that NK-2B is a transcription activator. NK-2B activates transcription from the promoter containing canonical NK-2 binding sites as well as from its own promoter, being expressed in cell culture. Experiments with transgenic flies show that endogenous NK-2B also activates transcription of the transgene bearing NK-2 binding sites at the promoter region.

The deletion of amino acids encoded by exon 1B strongly reduces the ability of NK-2B to activate transcription from the promoter containing canonical NK-2 binding sites. On the other hand, a single exon 1B fused to the HD of NK-2 strongly activates transcription of the reporter gene, suggesting that it constitutes the novel activation domain.

This activation domain in NK-2B is substituted for the repression domain mapped to the first exon of NK-2A (16). The removal of the repression domain and surrounding sequences may also unmask the potential activation region mapped to the second and third exons of the NK-2 protein or modify its secondary structure, thereby making an additional contribution to the transcription activation potency of NK-2B (16–19). Thus, the combined action of the activation region and the new N-terminal activation domain makes NK-2B a direct transcription activator. Interestingly, although the TN domain accounting for the interaction of NK-2 with co-repressor Groucho remains intact in NK-2B, it does not abolish its activation potency, since NK-2B in transgenic flies has proved to act as transcription activator even *in vivo*. In line with this fact are the results showing that the interaction with Groucho only slightly enhances the repressor activity of NK-2A (16–19).

Although NK-2A was shown to be a direct repressor (19), the question still arises as to its probable involvement in transcription activation. In particular, this may follow from the facts that mutation in *vnd/NK-2* leads to failure in activation of some proneural genes in *Drosophila* embryos and that NK-2A interacts with several other transcription factors (25). However, there is no evidence that NK-2A can directly activate transcription. It appears that *vnd/NK-2* has an indirect effect on the expression of proneural genes as a negative regulator of their repressors, or acts as a docking protein for activators. On the whole, we consider that NK-2A operates as a direct transcription repressor at all stages of fly development.

In addition to differences in the amino acid sequences of N-terminal domains between the two Nk-2 isoforms, differences in their structure (globular structure predicted for NK-2B and disordered one for NK-2A) should have a significant effect on their folding and their ability to interact with other proteins and DNA. In our experiments, NK-2A (in contrast to NK-2B) did not bind to the NK-2B promoter of the reporter vector or of the endogenous NK-2B gene. These results suggest that NK-2A apparently plays no significant role in the regulation of NK-2B expression. Indeed, while NK-2A expression is restricted to a particular region of the embryonic CNS, NK-2B is repressed in all cells of embryo, providing evidence that there must be an NK-2A-independent mechanism of NK-2B regulation. The interaction of NK-2B and NK-2A in *Drosophila* development deserves special study.

The results of our study show that the *vnd/NK-2* gene has a complex expression pattern that changes during the fly's life. This pattern is accounted by two alternative promoters activated in a stage- and tissue-specific manner. Activation of the NK-2A promoter takes place at early stages of embryonic development, in the precursors of nervous cells, and its activity is subsequently detected in neurons of the larval CNS and in the adult fly lamina.

Activation of the NK-2B promoter correlates with the onset of metamorphosis (in the larval period); its activity manifests itself in the gut and differentiating photoreceptor cells of the eye imaginal disk. One may suggest that the proteins of ecdysone cascade have an important role in NK-2B activation. Indeed, the existence of nine canonical binding sites for proteins encoded by *Broad-Complex (BR-C)* upstream of NK-2B promoter strongly supports this suggestion. The *BR-C* is an early ecdysone response gene that plays an essential role in controlling ecdysone-regulated gene expression during metamorphosis (23).

In the adult fly, the NK-2B promoter is active in the retina, lamina and gut cells. The start of NK-2B expression in photoreceptors of the eye imaginal disk correlates with the onset of their differentiation and continues in differentiated photoreceptor neurons of the adult eye. Therefore, NK-2B is involved in the chain of genetic events leading to differentiation of neurons and subsequent maintenance of their functioning. Moreover, NK-2B as well as NK-2A is expressed in the lamina, indicating a role for both protein isoforms encoded by the *vnd/NK-2* gene in the maintenance of the *Drosophila* visual system.

In contrast to NK-2B, whose expression correlates with the onset of neuronal differentiation, NK-2A is expressed mainly in undifferentiated neurons. In larvae, NK-2A was found in neuroblasts and their cell progeny, which remain undifferentiated until the pupa stage. The neuroblasts producing the postembryonic CNS appear to be the same as those that give rise to the larval CNS in embryos. The stem cell mode of their division is similar to that observed during formation of the larval brain. As in the embryo, larval neuroblasts continue to express NK-2A; moreover, this expression is confined to a particular CNS region, indicating that NK-2A may be essential for the formation of the corresponding part of the adult

brain. No expression of NK2A was detected in the adult fly brain. Thus, it appears that the two NK-2 isoforms generally have opposite functions in *Drosophila* development: NK-2B is essential for neuronal differentiation, while NK-2A maintains the undifferentiated status of neuroblasts.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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