Distinct features of neurotransmitter systems in the human brain with focus on the galanin system in locus coeruleus and dorsal raphe

Erwan Le Maître[a,1,2], Swapnali Shantaram Barde[a,1,2], Miklos Palkovits[b,c], Rochellys Diaz-Heijtz[a], and Tomas G. M. Hökfelt[a,2]

[a]Department of Neuroscience, Karolinska Institutet, 17177 Stockholm, Sweden; and [b]Neuromorphological and Neuroendocrine Research Laboratory, Semmelweis University, and [c]Hungarian Academy of Sciences, 1094, Budapest, Hungary

Contributed by Tomas G. M. Hökfelt, December 8, 2012 (sent for review September 8, 2012)

Using riboprobe in situ hybridization, we studied the localization of the transcripts for the neuropeptide galanin and its receptors (GalR1–R3), tryptophan hydroxylase 2, tyrosine hydroxylase, and nitric oxide synthase as well as the three vesicular glutamate transporters (VGLUT 1–3) in the locus coeruleus (LC) and the dorsal raphe nucleus (DRN) regions of postmortem human brains. Quantitative real-time PCR (qPCR) was used also. Galanin and GalR3 mRNA were found in many noradrenergic LC neurons, and GalR3 overlapped with serotonin neurons in the DRN. The qPCR analysis at the LC level ranked the transcripts in the following order in the LC: galanin >> GalR3 >> GalR1 > GalR2; in the DRN the ranking was galanin >> GalR3 >> GalR1 = GalR2. In forebrain regions the ranking was GalR1 > galanin > GalR2. VGLUT1 and -2 were strongly expressed in the pontine nuclei but could not be detected in LC or serotonin neurons. VGLUT2 transcripts were found in very small, nonpigmented cells in the LC and in the lateral and dorsal aspects of the periaqueductal central gray. Nitric oxide synthase was not detected in serotonin neurons. These findings show distinct differences between the human brain and rodents, especially rat, in the distribution of the galanin system and some other transmitter systems. For example, GalR3 seems to be the important galanin receptor in both the human LC and DRN versus GalR1 and -2 in the rodent brain. Such knowledge may be important when considering therapeutic principles and drug development.

noradrenaline | species difference | transmitter coexistence | monoamines | depression

The locus coeruleus (LC) and the dorsal raphe nucleus (DRN)/raphe median nucleus (MRN) have been the focus of clinical and preclinical monoamine research for almost half a century. Using the formaldehyde fluorescence (Falck–Hillarp) method (1), Dahlström and Fuxe (2) originally described these nuclei in the rat as containing noradrenaline (NA) (the A6 group) and 5-hydroxytryptamine (5-HT; serotonin) (the B7/8 groups), respectively. The LC harbors 2,800–3,600 neurons with an additional 260 neurons in the subcoeruleus area, the great majority of which are noradrenergic (3–6).

The DRN forms a rostro-caudal, ventral midline column and is part of the periaqueductal central gray (PAG) (7) with a large number of 5-HT neurons that can be subdivided into several subgroups (2, 8–11). Both the NA-LC (5, 6, 12) and the 5HT-DRN (8–11, 13) neurons have wide projections to most forebrain areas.

In humans the LC is a compact, blue-pigmented nucleus consisting of a total of ∼50,000 neurons (both sides), almost all of which are noradrenergic (14–16). The DRN comprises about 165,000 5-HT neurons, which constitute around 70% of all DRN neurons (17). Thus, there are numerous nonserotonergic neurons in the human DRN, as is also the case in other species, including the mouse (18, 19).

It now is well established that most neurons synthesize and release several types of messenger molecules in the process known as "cotransmission" (20–26). For example, in the rat some serotonergic DRN neurons synthesize nitric oxide (NO), visualized as NO synthase (NOS) and/or NADPH-diaphorase (27–32), and glutamate, visualized as vesicular glutamate transporter 3 (VGLUT3) in both the rat (33–38) and mouse (19, 39). Many neuron systems have one or more coexisting neuropeptides; for example, galanin is present both in the NA-LC and the 5HT-DRN neurons in the rat (40).

Galanin, a 29-aa neuropeptide (41) [30 aa in humans (42–44)] is widely distributed in the rodent brain (45–50) and exerts its effects via G protein-coupled galanin receptors (51–55) with a wide distribution in rodents (39, 56–60). So far, three of these receptors have been identified, GalR1–3.

In the rat galanin coexists with NA in most LC neurons (40, 61, 62) and with 5-HT in many DRN neurons (32, 40). It is highly likely that galanin also is coexpressed in the NA-LC neurons of the mouse (39, 45, 49) and human (63–65). However, mouse 5HT-DRN neurons do not synthesize galanin (19, 39, 45, 49, 66), nor has galanin expression been detected in human DRN neurons (64).

Early autoradiographic studies showed binding of iodinated galanin to LC and DRN in the rat (67–69) and primates, including humans (70–72). In situ hybridization (ISH) has demonstrated the GalR1 and -2 transcripts in the rat LC and GalR1 in the ventrolateral PAG; the relationship to the 5HT-DRN neurons is not clear, although a weak GalR2 mRNA signal was observed in the midline (56, 58, 73). These results generally were in agreement with the quantitative real-time PCR (qPCR) results (60). How-

Significance

For decades rodents have been used to explore normal brain functions and mechanisms underlying brain diseases. Such data often have been the basis in the search for new drugs. In this study we selected chemical markers associated with central noradrenaline and serotonin neurons, key systems in research on and current treatment of depression, and studied their expression with in situ hybridization in postmortem human brains. The results show some distinct species differences between human and rodent noradrenergic and serotonergic neurons which may better inform the development of novel anxiolytic/antidepressant drugs.

Author contributions: E.L.M., S.S.B., R.D.-H., and T.G.M.H. designed research; E.L.M., S.S.B., and R.D.-H. performed research; M.P. contributed new reagents/analytic tools; E.L.M., S.S.B., R.D.-H., and T.G.M.H. analyzed data; and E.L.M., S.S.B., R.D.-H., and T.G.M.H. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

[1]E.L.M., and S.S.B. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: Erwan.LeMaitre@ki.se or tomas.hokfelt@ki.se.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221378110/-/DCSupplemental.

ever, none of the galanin receptor subtypes have been identified so far in the human LC or DRN.

In this study we analyzed sections/slices including the LC and DRN in human postmortem brains using ISH and riboprobes generated to identify galanin and GalR1-3. To identify NA and 5-HT neurons, probes were designed for the human catecholamine-synthesizing enzyme tyrosine hydroxylase (TH) (74, 75) and the 5-HT–synthesizing enzyme tryptophan hydroxylase 2 (TPH2), respectively (76). A limited qPCR analysis of these markers was carried out at the pontine level and, for the galanin system, in some forebrain areas also.

Finally, we attempted to identify a relationship between NA and 5-HT neurons, on the one hand, and between NOergic and glutamatergic neurons on the other hand, using probes against nNOS (77, 78) and the three VGLUTs (79–81), respectively. Preliminary results from these studies have been reported at meetings (82, 83).

Results

ISH. The LC/subcoeruleus is a bilateral compact accumulation of neurons in the pons with a limited rostro-caudal extension and a characteristic localization close to the fourth ventricle. This nucleus is characterized by the expression of the catecholamine-synthesizing enzyme TH, here visualized through the TH transcript (Fig. 1 *A* and *B* and Fig. S1*A*). In the dark-field configuration the NA neurons can be distinguished by their autofluorescence (Fig. 1*H*). Therefore, in several instances we used the pigment as a marker for NA-LC neurons instead of using TH ISH. In contrast, the 5-HT neurons expressing the TPH2 transcript in the caudal mesencephalon/pons (Fig. 2 *A* and *D* and Fig. S1*B*) extend over a long distance with characteristic distribution patterns at different levels (Fig. S1*C*). This group is localized in the midline ventral to the aqueduct, extending laterally in two wings in the ventral PAG (vPAG) and continuing further ventrally as MRN with many neurons dispersed in the pontine reticular formation (Fig. S1 *B and C 1–5*).

The NA-LC neurons express robust levels of TH, which can be observed after comparatively short exposure times (Fig. 1 *A* and *B*) and are overexposed after only 10 d, as compared with the less strong expression of galanin) (4-wk exposure time) (Fig. 1 *C* and *D* and the weakly expressed GalR3 (8-wk exposure time) (Fig. 1 *E* and *F*). Thus, it is the varying exposure times that make the signals in Fig. 1 *B*, *D*, and *F* look approximately similarly strong. Note that GalR3 transcript levels are fairly similar among individual neurons (Fig. 1*F*), as is the TH signal (Fig. 1*B*), in contrast to the high variability of galanin mRNA (Fig. 1*D*). GalR3 appears to be the predominant galanin receptor in the LC (Fig. 1*G*). A weak GalR1 signal was seen in the LC region expanding medially into the pontine central gray but did not seem to be associated with the pigment characteristic of the NA

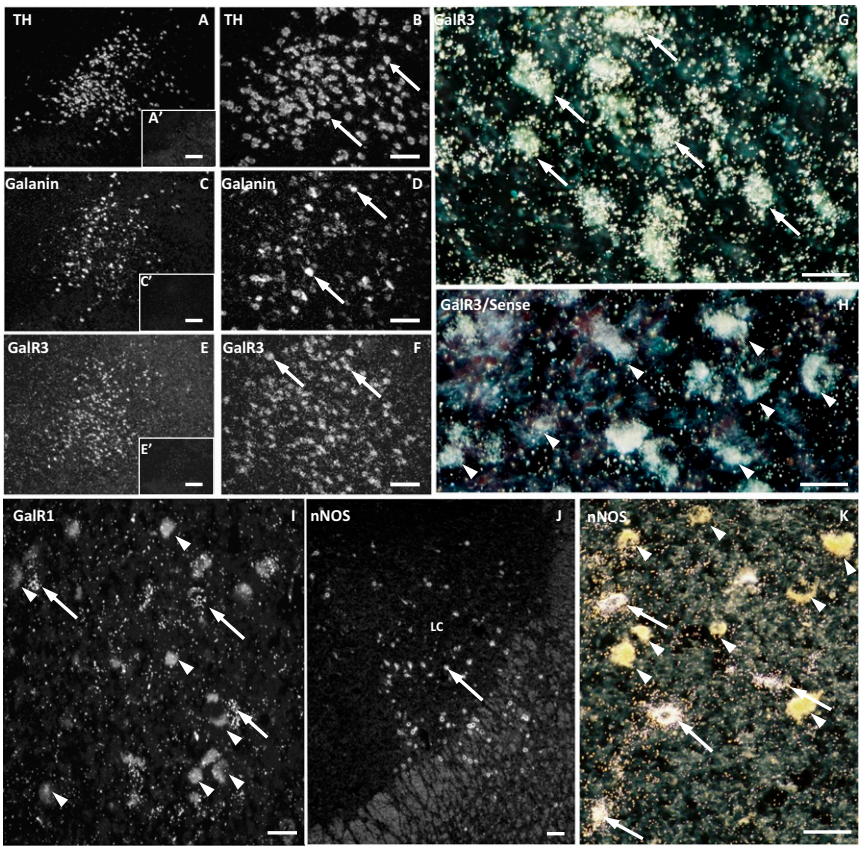

Fig. 1. Dark-field ISH photomicrographs showing the distribution of galanin, GalR1, GalR3, and nNOS in the LC. Autofluorescent pigmented neurons are indicated by arrowheads, and positive cells are indicated by arrows. The three markers TH (*A* and *B*), galanin (*C* and *D*), and GalR3 (*E* and *F*) show an overlapping distribution pattern, whereby TH and GalR3 transcript levels are similar in all cells. Note the variability in the strength of the signal for galanin mRNA. *Insets* A', C', and E' show results after hybridization with sense/control probe. Strong signal for GalR3 is seen in LC (*G*) but not after hybridization with the control probe (*H*). Note bluish autofluorescence from pigment in NA-LC neurons (arrowheads in *H*). (*I*) A very weak signal for the GalR1 transcript is seen in the LC, but it is probably not present in the NA neurons. nNOS mRNA overlap with TH^+ cells in the LC only to a minor extent in its most rostral part (*J*), where pigmented neurons intermingle with NOS^+ neurons (*K*). Exposure time: TH, 10 d; galanin, 4 wk; GalR1 and GalR3, 8 wk; nNOS, 6 wk. (Scale bars: 200 μM in *A*, *C*, *E*, and *J*; 100 μM in *B*, *D*, and *F*; 50 μM in *I* and *H*; 25 μM in *G* and *K*.)

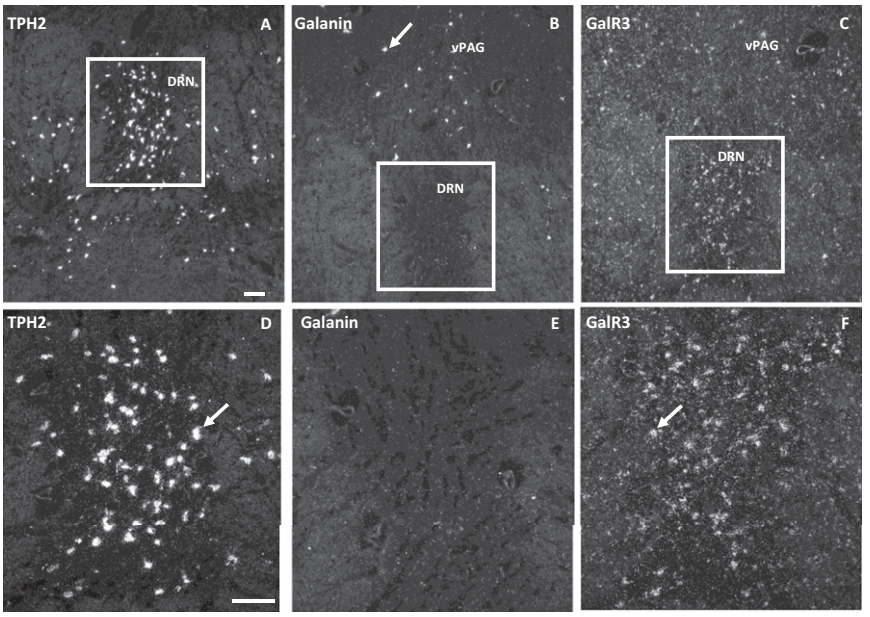

Fig. 2. Dark-field ISH photomicrographs showing the distribution of TPH2 (*A* and *D*), galanin (*B* and *E*), and GalR3 (*C* and *F*) in semiadjacent sections. *D*, *E*, and *F* show higher magnification of boxes in *A*, *B*, and *C*, respectively. Arrows indicate cells positive for the respective marker. Note overlapping distribution of TPH2 and GalR3 in the posterior DRN (compare *D* and *F*). However, no galanin signal is seen in that particular area (*B* and *E*). Instead galanin+ cells are seen more dorsally (*B*). Exposure time: TPH2, 10 d; galanin, 4 wk; GalR3, 8 wk. (Scale bars: 100 μM in *A*, *B*, and *C*; 50 μM in *D*, *E*, and *F*.)

neurons (Fig. 1*I*) as confirmed in cresyl violet-counterstained sections analyzed with bright- and dark-field microscopy (see Fig. S3 *A* and *B*).

A comparison of the distribution of transcripts for TPH2 (Fig. 2 *A* and *D*), galanin (Fig. 2 *B* and *E*), and GalR3 (Fig. 2 *C* and *F*) in the DRN/vPAG shows that TPH2 and GalR3 exhibit a distinct overlap, but no galanin mRNA can be seen in this particular subregion (Fig. 2 *B* and *E*). (Exposure times for TH, galanin, and GalR3 were approximately as stated above.) However, galanin mRNA is observed at this level just dorsal to the serotonin cells and extending dorsally/laterally in the vPAG (Fig. 2*B*). Galanin mRNA was also seen in a number of additional nuclei, including the inferior colliculus (Fig. S2 *A* and *D*), the tegmental peduncular pontis (Fig. S2 *B* and *E*), the medial parabrachial nucleus (Fig. S2 *C* and *F*), and the subcuneiform nucleus (Fig. S2 *G* and *H*). GalR1 and -3 were also observed in other regions in the sections analyzed; e.g., a robust GalR1 signal was observed in the lateral PAG (Fig. S3*C*), and GalR3 signal was seen lateral to the aqueduct. A signal for GalR2 mRNA could not be detected in any of the sections.

nNOS was expressed in many regions of the brainstem, but these cells overlapped with TH-positive (+) mRNA in the LC only to a minor extent (Fig. 1*J*) and only in its most rostral part, where pigmented NA neurons intermingled with NOS^+ neurons (Fig. 1*K*). There was no overlap with the $TPH2^+$ 5-HT neurons in the DRN or MRN (compare *A* and *B* in Fig. 3). However, strongly labeled large neurons were seen in the ventro-lateral PAG extending into the reticular formation, and many small, weakly labeled cells were encountered in the dorso-lateral region of the PAG.

VGLUT1 mRNA could not be detected in the vPAG, including the DRN (Fig. 4*A*). However, a very strong VGLUT1 mRNA signal was observed in many neurons of the pontine nuclei (Fig. 4 *B* and *C*). VGLUT2 also was strongly and frequently expressed in these nuclei, overlapping with VGLUT1 mRNA. At the level of LC, VGLUT2 mRNA was seen in the pontine central gray substance, in the LC area, and extending medially into the region of the dorsal tegmental nucleus (Fig. 4*D*). High-power magnification showed that in most instances grains did not appear to overlie the autofluorescent noradrenergic cell bodies but instead represented a separate population of very small cells, possibly glial cells (Fig. 4*E*). In contrast to VGLUT1, there was a strong VGLUT2 signal in the PAG, primarily in the lateral/dorsolateral region (Fig. 4 *G* and *H*). The midline area lacked detectable transcript, in strong contrast to the distribution of TPH2 mRNA (compare *F* and *G* in Fig. 4).

qPCR. The relative expression of galanin and its receptors was analyzed by qPCR in tissue slices of containing LC and DRN and in macrodissected tissue punches from frontal cortex, cingulate cortex, and the amygdaloid complex (for details, see *SI Materials and Methods*). In addition, TH [raw cycle threshold (Ct) value 28] and TPH2 (raw Ct value 25) mRNA levels also were analyzed in such slices (Table S1). Galanin mRNA expression was ~42-fold higher than GalR1 in the LC slices and was ~25-fold higher than GalR1

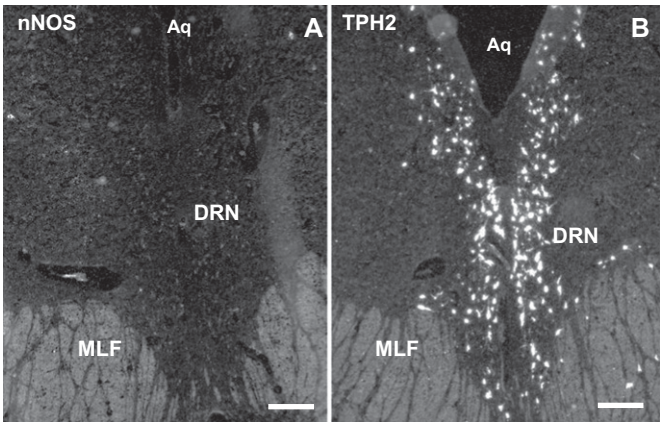

Fig. 3. Dark-field ISH photomicrographs showing the distribution of nNOS (*A*) and TPH2 (*B*) transcripts in semiadjacent sections. There is no overlap with the $TPH2^+$ 5-HT neurons in the DRN. Aq, Aqueduct; MLF, medial longitudinal fascicle. Exposure time: TPH2, 10 d; nNOS, 6 wk. (Scale bars: 100 μM.)

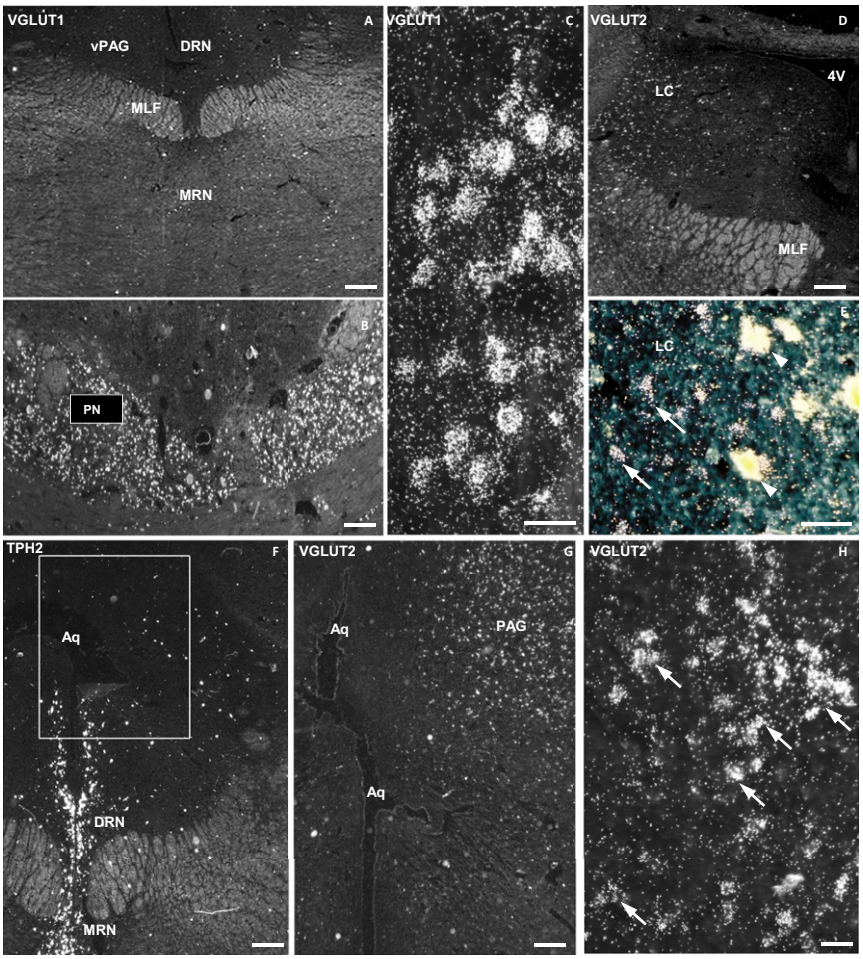

Fig. 4. Dark-field ISH photomicrographs showing the distribution of VGLUT1 (*A*–*C*), VGLUT2 (*D*, *E*, *G*, and *H*), and TPH2 (*F*) mRNA at the pontine level. The box in *F* shows approximately the region displayed in the semiadjacent section in *G*. There is no detectable signal for VGLUT1 in the vPAG, including the DRN (*A*), whereas there is a very strong signal for both VGLUT1 (*B*) and -2 (*C*) in the pontine nuclei. A distinct VGLUT2 signal is seen in the pontine central gray, including the LC, extending medially to the dorsal tegmental nucleus (*D*). As seen in the high-power magnification, the cells are very small (arrows) compared with the pigmented NA neurons (arrowheads in *E*). Numerous vGLUT2 mRNA+ cells are observed in the lateral PAG (*G* and *H*), although there are very few cells in the DRN and vPAG (compare *G* and *F*). MLF, medial longitudinal fascicle; PN, pontine nuclei. Exposure time: TPH2, 10 d; VGLUT1 and VGLUT2, 8 wk. (Scale bars: 200 μM in *A*, *D*, and *F*; 100 μM in *B* and *G*; 50 μM in *H*; 25 μM in *C* and *E*).

in the DRN slices (Table S1). GalR3 was the most abundantly expressed galanin receptor, being approximately sixfold higher than GalR1 in LC slices and approximately sevenfold higher in DRN slices. GalR2 was expressed at very low levels (Fig. 5). The raw Ct values for galanin and GalR1-3 transcripts are given in Table S1.

In the forebrain regions, however, GalR1 was the most abundantly expressed receptor, even higher than galanin. Galanin mRNA was ∼0.6-fold, ∼0.4-fold, and ∼0.8-fold lower than GalR1 in the frontal cortex (Fig. S4*A*), cingulate cortex (Fig. S4*B*), and amygdaloid complex (Fig. S4*C*), respectively. However, it is possible that GalR2 and GalR3 were not expressed, because the Ct values were >35 for both. Taken together, these results indicate that GalR3 seems to be the abundantly expressed receptor in the brainstem nuclei studied here, whereas GalR1 appears to predominate in the forebrain regions.

Controls. Hybridization with control probes resulted in a complete absence of signals for TH (Fig. 1*A′*), galanin (Fig. 1*C′*), and GalR3 (Fig. 1 *E′* and *H*) in the LC.

Discussion

Almost 30 y ago Tatemoto, et al. (41) discovered galanin, and subsequently three receptors were identified, GalR1-3 (51, 53, 55), which are the focus of the present study. Here we studied these molecules in selected areas of human postmortem brains with ISH and qPCR to establish expression patterns and to reveal possible differences among species. Some other transmitter-related markers, i.e., TH, TPH2, nNOS, and VGLUTs, were included also.

The main results of the present study are that, in humans, (*i*) galanin is present in NA-LC neurons but not in 5-HT-DRN neurons; (*ii*) GalR3 is the major galanin receptor in NA-LC neurons and probably in 5-HT-DRN neurons; (*iii*) 5-HT-DRN neurons do not express nNOS; (*iv*) GalR2 is expressed at low levels, if at all, in NA-LC and 5-HT-DRN neurons; (*v*) GalR3 probably is not expressed in the forebrain regions studied; instead, GalR1 is the predominant receptor; (*vi*) VGLUT2 is expressed only in the dorsal and lateral PAG; and, of special importance, (*vii*) distinct differences exist among species (Table 1).

It should be noted that we report only transcripts without evidence for the expression of the receptor protein. Thus, GalR2 and -3 receptors may be present on afferents (presynaptic) to the forebrain regions studied. For example, there is evidence that in the rat the NA-LC neurons synthesize GalR2, which probably is transported centrifugally to act as a presynaptic receptor in forebrain regions (84).

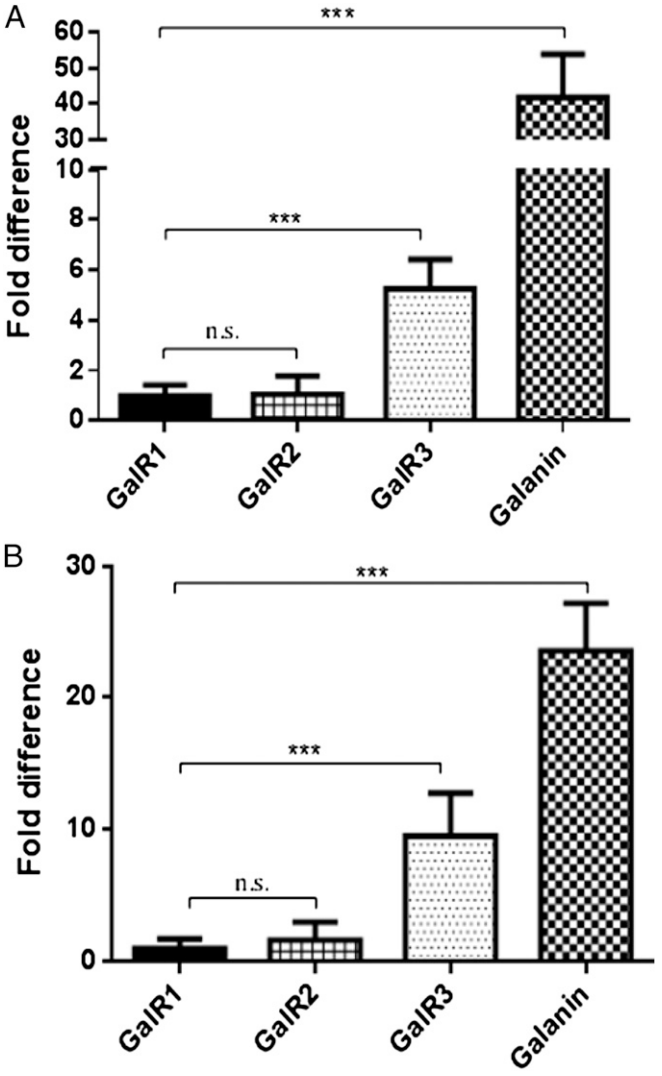

Fig. 5. qPCR was used to examine the mRNA levels of galanin, GalR1, GalR2, and GalR3 in the LC (*A*) and DRN (*B*). The expression level for each gene examined was normalized to *β-actin* and then expressed relative to GalR1, the value for which was set at 1. Expression levels for galanin and GalR3 in the LC and DRN are significantly higher than those of GalR1 and GalR2, which are very low. The bar graphs represent the mean ± SEM (n = 7). Statistical significance was determined using unpaired Student's t test. *P < 0.05; **P < 0.01; ***P < 0.001; n.s., not significant.

Galanin System: LC. The present results confirm that many neurons in the LC in the human brain robustly express galanin and TH (for references, see the Introduction). The analysis was facilitated by the fact that the NA-LC neurons express a distinct pigment and thus do not need to be identified by TH staining. Importantly, a strong GalR3 signal but no GalR1 signal was detected in these neurons. However, a weak GalR1 mRNA signal was seen in non-NA cells in the LC and in some other brain regions, apparently, the GalR2 transcript could not be detected. In agreement, the qPCR results confirmed that the GalR3 transcript was higher than GalR1 and GalR2 at the LC level. Galanin itself was robustly expressed.

The marked variation in galanin mRNA levels among individual LC neurons, in contrast to the apparently similar levels of GalR3 (and TH), probably reflects the fact that galanin is a released molecule that must be replaced through compensatory synthesis, i.e., requires new transcript. This variation also may indicate that individual LC neurons have different activities/functions. GalR3, on the other hand, like the enzyme TH, is a comparably stable protein, a receptor with a low turnover rate.

The GalR3 result was surprising because, based on ISH, GalR1 and -2, but apparently not GalR3, are present in the rat and mouse LC (39, 56–59). In fact, GalR3, the last cloned receptor (85, 86), has a comparatively limited expression in the rat brain (57, 60). The *Allen Brain Atlas* provides no information about GalR3 in the mouse brain (39), possibly reflecting absence or low levels. Whether our riboprobe(s) that were selective for GalR2 failed or whether GalR2 indeed is not present in the human LC remains to be clarified. In the qPCR analysis of LC, GalR2 had the highest Ct value, suggesting a very low, if any, expression (see *Methodological Aspects* below).

Galanin System: vPAG/DRN. Some galanin cells with a fairly weak signal were seen in the midline and in the vPAG but extended mainly dorsolaterally and did not overlap with the 5-HT neurons. Numerous galanin+ neurons also were seen in the same sections, e.g., in the inferior colliculus and other regions. These results and the LC data suggest that the galanin probe was functional. In contrast, there was a strong overlap between 5-HT and GalR3 mRNA$^+$ neurons, indicating coexistence. However, we failed in attempts to carry out double-ISH to provide final evidence for coexistence. One explanation for the failure may be that the exposure time to obtain a robust GalR3 signal was more than 2 mo. Taken together, the results indicate that in humans galanin is expressed strongly in NA-LC neurons but not in 5HT-DRN neurons, similar to expression in the mouse but in contrast to expression in the rat. The LC and probably the DRN express GalR3 in humans, thus representing targets for GalR3 antagonists/agonists. The qPCR data agree with the ISH results, with galanin being the most abundantly expressed molecule, followed by GalR3 and then GalR1 and -2.

Galanin System: Forebrain. A limited qPCR analysis of the transcripts for the galanin systems was carried out in two cortical regions and the amygdala. Interestingly, the levels of all markers were generally low [raw Ct values ranged from ~40 (GalR3) to ~31 (GalR1)]; the GalR1 transcript showed the highest levels, even higher than galanin itself. Thus, the early ^{125}I-galanin binding studies on some monkey and human forebrain areas (70–72) might have visualized mainly GalR1 receptors, although receptors on afferents represent a possibility (see above). These results indicate an interesting situation: GalR3 is associated with projection neurons in the lower brainstem but may be of little importance in the forebrain, where GalR1 seems to predominate. It is likely that GalR1 and perhaps GalR3 are postsynaptic receptors (84).

Differences Among Species in Other Galanin Systems. Differences among species have been noted for other galanin systems. In the rat the cholinergic forebrain neurons, of interest in relation to Alzheimer's disease (87), express galanin after the inhibition of axonal transport with colchicine (88, 89) and after brain injury (90–92), although the galanin transcript can barely be detected in these neurons in the normal rat (93). Colchicine also is needed to detect galanin peptide in these neurons in mouse (49). However, strong expression can be seen in some monkeys [e.g., owl monkeys (94), capuchin monkeys and rhesus monkeys (95)] but not in humans. Thus, with regard to this system, the rat seems to be more similar to humans than to the lower monkeys. To what extent human cholinergic forebrain neurons have the capacity to express galanin, as seen in the rat and the mouse, is still unclear. As a note, Chan-Palay et al. (96) reported the presence of galanin mRNA$^+$ neurons in the human forebrain, but these neurons probably were not identical to the cholinergic neurons. Nevertheless, Mufson and colleagues (97) have reported impressive

Table 1. Galanin and its receptors and NOS are differentially expressed in NA (LC) and 5-HT (DRN) neurons of three species

Species	Galanin LC/DRN	GalR1 LC/DRN	GalR2 LC/DRN	GalR3 LC/DRN	nNOS LC/DRN
Human	++++/0	(+)/0	n.d./n.d.	+++/+++	++/0
Rat	++++/++	++/0	++/+	0/0	0/+++
Mouse	++++/0	n.d./n.d.	++/n.d.	n.d./n.d	0/0

Subjective rating ranging from weak (+), medium strong (++), strong (+++), to very strong (++++) signal. n.d., not detected. Data are from the present study and papers cited in the text.

data providing evidence that galanin has a protective role in Alzheimer's disease.

A similar situation may exist in the histaminergic/GABAergic tubero-mammilary neurons that express galanin after colchicine administration in the rat (48, 98–100) but apparently not in human, although this analysis was performed only with immunohistochemistry and not with ISH (101).

nNOS and Glutamate. Neither nNOS nor VGLUT3 could be detected in human 5-HT neurons. The lack of nNOS in human is similar to mouse (19, 39) and different from rat (27–30, 32) but is in agreement with a study by Carrive and Morgan (102) based on NADPH-d histochemistry showing strong staining in the dorsal PAG but apparently none in the DRN. [NAPDH-d in the rat has been shown to be identical to nNOS (103, 104)]. Here we provide confirmatory evidence by showing the apparent absence of nNOS transcript in human 5-HT neurons based on ISH but a strong signal in the nearby regions, indicating a working probe. Also the NA-LC neurons had no detectable nNOS signal, even if $nNOS^+$ cells intermingled with NA neurons to a limited extent.

The discovery of VGLUTs (79–81) made it possible, via immunohistochemistry and ISH, to identify unequivocally the neurons using the excitatory amino acid glutamate as transmitter. However, even though powerful antibodies to the VGLUTs have been generated, VGLUT expression in neuronal cell soma can be visualized only with ISH, because antibodies apparently show the transporters only in nerve endings. [DRG neuron cell bodies are an exception (105, 106).] The distribution patterns in mouse and rat DRN/PAG are similar (for references, see Introduction): no VGLUT1 mRNA is found in the LC, DRN, or vPAG. There are many VGLUT2 $mRNA^+$ cell bodies in the rat and mouse vPAG but not in the LC or in the midline of the vPAG, i.e., the DRN. In contrast, a distinct subpopulation of 5-HT neurons in the DRN express VGLUT3 transcript in rats (33–35), mice (39), and syrian hamsters (107). However, not all rat $VGLUT3^+$ neurons in the DRN are serotonergic (34).

In humans, as in rats and mice, no VGLUT1 transcript was found in the LC region or in the vPAG. VGLUT2 mRNA was found in small cells (possibly glia) in the LC, partly intermingling with NA neurons, and was seen only in the dorsal and lateral PAG, in contrast to the distribution throughout the entire PAG in rodents. The lack of a VGLUT3 signal may represent either a false negative caused by an unsensitive or failed probe or a true absence. Thus, it still is uncertain whether VGLUT3 expression in the 5-HT neurons in the vPAG differs in rodents and humans.

Comparison with Results from the Allen Institute for Brain Research. Scientists at the Allen Institute for Brain Research also have explored the human brain (108, 109), as described in *SI Discussion*. The data analysis revealed some individual variability, but, in general, galanin expression was higher in the supraoptic nucleus, hypothalamus, LC, and frontal lobe than in the central nucleus of the amygdala and MRN. TH and TPH2 transcripts also were highly expressed in LC and MRN, respectively. Expression levels for the galanin receptors were generally low and are difficult to compare with the present qPCR and ISH results. Taken together, the results from the Allen Institute are, to a certain extent, in agreement with the qPCR and ISH results in our study. However, this comparison needs further, in-depth analysis. Non-isotopic ISH for galanin and GalR2 also has been performed in cortical and subcortical regions, where a weak signal is observed for both transcripts (109).

Methodological Aspects. Histochemical approaches always are associated with methodological problems related to sensitivity and specificity (110, 111). These difficulties are accentuated when studying human postmortem tissue because of variability among human beings, deterioration of the tissue with postmortem time, varying conditions of death, and subsequent handling. Even more problematic are low-abundance transcripts, e.g., for certain receptors. Such proteins have slow turnover and thus low mRNA levels because they, unlike neuropeptides, do not need to be replaced swiftly after release. Presumably such receptors can be revealed only by advanced molecular biology combined with electrophysiology (112, 113).

In the present study, the transcripts for robustly expressed molecules, such as TH and TPH2, could be demonstrated in all brains but, importantly, after considerable differences in exposure time to an autoradiographic film/emulsion. Thus, although sections from some brains hybridized for TH or TPH2 needed less than a week of exposure, sections from other brains had to be exposed for several weeks. Not unexpectedly, such differences were critical for the successful processing of the low-abundance galanin receptor transcripts. Thus, in principle, such transcripts could be detected only in brains with short exposure time for TH and TPH2. Therefore, negative results should be interpreted with caution.

The probe also is essential. We obtained good results for GalR3 with only one of the designed probes. The probe may not be as good for GalR1 for GalR3, and the probe may have failed completely for GalR2 and VGLUT3. Again, qPCR results indicate very low GalR1 levels. As a note, GalR2 and -3 have a high degree of homology; therefore in a previous study we demonstrated the specificity of the primers for the two receptors (114).

Difficulties in detecting galanin receptor transcripts are supported by the recently published *Allen Brain Atlas* describing the distribution of some 20,000 transcripts throughout the mouse brain (39): No results are reported for GalR3, GalR2 is distinctly expressed only in some of the total of 52 sections/levels, and GalR1 appears less abundant than in rat brain. However, the presence of the GalR3 transcript has been reported in many rat brain regions by Mennicken et al. (57) using ISH and by Waters and Krause (60) using qPCR. In contrast, the *Allen Brain Atlas* shows robust expression of many other neuropeptide receptors and thus supports our view that galanin receptor transcripts may be particularly difficult to visualize with ISH and, as shown here, probably are even more difficult to visualize in human postmortem tissue.

The qPCR analysis was carried out on full coronal sections of the pons, from a level including either the DRN or LC; in fact, these sections were alternate sections to those taken for ISH (*SI Materials and Methods*). However other regions within the slice expressing the studied markers to a varying extent have been included also. For the LC level, TH probably is confined to the

LC and subcoeruleus; GalR1 and -3 were observed only in relation to the LC, whereas galanin was expressed in several other nuclei (Fig. S2). TPH2 is fairly widely expressed both in the DRN and MRN and in the reticular formation. Galanin and GalR1 also are expressed outside the raphe region, but we observed GalR3 only in the DRN and lateral to the aqueduct. Thus TH mRNA is diluted, and results for galanin and GalR1 are not selective for the LC region. In fact, a preliminary qPCR experiment with laser capture microdissection (LCM) of the NA-LC nucleus (defined by pigmented neurons) shows a higher level of TH transcript expression (i.e., a raw Ct value of ∼22 for LCM versus ∼28 for the whole section). Similarly for GalR3 the raw LCM Ct value was ∼29, versus ∼33 for the whole section, thus showing the expected dilution effect for these markers.

Autoinhibition, Dendro-Somatic Release, and Electrophysiology. Autoinhibition of LC neurons mediated by NA (115–117) and of DRN neurons mediated by 5-HT (118) may involve not only release from collaterals but also somato-dendritic release (119, 120). This type of release also has been shown for neuropeptides (121) and possibly for galanin in the LC (122). It is assumed that this autoinhibitionis responsible, at least in part, for the delayed onset of the clinical effect of monoamine-reuptake inhibitors (123, 124).

Electrophysiological studies in the rat have revealed that galanin hyperpolarizes NA-LC neurons (68, 125, 126), presumably mediated via GalR1 (84), a transcript known to be present in these neurons (56, 58). In addition, galanin at low concentrations (10^{-9}M) enhances the autoinhibitory effect exerted by NA on LC neurons via α2A adrenoreceptors (127). Moreover, GalR1, but not -2 or -3, is regulated by galanin signaling in the rat LC (128, 129). Galanin also has an inhibitory effect on some 5-HT neurons in the DRN. However, it still is unclear if these effects are direct, via GalR1 (73) or GalR3 (130, 131), indirect via GABA neurons (132), or both. ISH results indicate that GalR1 indeed is present in the vPAG but not in 5-HT neurons (56, 58, 73).

Moreover, low galanin concentrations enhance the autoinhibitory effect of 5-HT via the 5-HT1A receptor in the DRN (73). This effect may be related to the formation of GalR1-5-HT1A receptor heteromers as recently reported by Borroto-Escuela et al. (133). Such receptor complexes increase trafficking of 5-HT1A receptors to the plasma membrane (133) and have been suggested to contribute to development of depression (134).

Neuropeptides and Stress-Related Disorders. Several antidepressants exert their effect via monoamine neurons (124, 135–137). It has been proposed, based on animal experiments over the last few decades, that neuropeptide also receptors are putative targets for development of antidepressants, including receptors for substance P, neuropeptide tyrosine, corticotropin-releasing factor/corticotropin-releasing hormone, melanocyte-concentrating hormone, vasopressin, and dynorphin (138–143), as well as the galanin system. Thus, galanin interacts with 5-HT1A receptors, and galanin antagonists and agonists have anxiolytic and antidepressive effects in animal experiments (144–151). Interestingly, association of genes encoding galanin and/or GalR3 has been reported for psychiatric phenotypes (152–154), including panic disorder (155), depression-related parameters (156–158), and nicotine dependence (159, 160).

The LC plays an important role in the development of stress-related disorders (161–169), and stress up-regulates galanin expression in the rat LC (170, 171). We therefore hypothesize that such disorders are associated with an increase in firing and with increased galanin synthesis and release from LC neurons, especially from soma and dendrites (68, 122), resulting in activation of GalR1 autoreceptors, inhibition of firing, and decreased NA release in the forebrain. Together, these events presumably result in a prodepressive effect. In theory, a similar scenario may be true for the 5-HT/galanin neurons in the DRN (73). Consequently, attenuating the inhibition and autoinhibition of NA and 5-HT neurons by galanin antagonists may have anxiolytic/antidepressive effects. In fact, these effects may be enhanced by the GalR1-5-HT1A heterodimerization described above. Finally and interestingly, Murck, et al. (172) have shown that the effects of i.v.-administered galanin on sleep EEGs in healthy subjects are similar to those seen with sleep deprivation (172) and that galanin, when given to patients with depression, has an acute antidepressive effect (173). The site of action of peripherally administered galanin remains to be analyzed.

Based on the rat experiments, a GalR1 antagonist would be suitable to obtain such an effect in the LC (56, 58, 61, 84), whereas a GalR3 antagonist (130, 131) may be more appropriate in the DRN. Our study shows that in the human brain the NA-LC, and presumably the 5-HT-DRN neurons, express GalR3, not GalR1 as in the rat (56, 58). The transduction mechanism for GalR3 has not been well characterized. Smith et al. (93) used *Xenopus* oocytes and coexpressed GalR3 with the potassium channel subunits GIRK1 and -4. They found that galanin opens potassium channels and thus hyperpolarize the cell membrane. If the result from this artificial system should turn out to hold true for human NA LC and 5-HT DRN neurons also, then a GalR3 antagonist could have the same effect in humans as in the rat.

The investigation of galanin has been hampered by the lack of selective and powerful pharmacological tools to analyze galanin functionality, particularly drugs that can penetrate the blood–brain barrier (174). However, a small number of such compounds active at the GalR3 receptor, with anxiolytic and antidepressant activity in various rat models, have been developed (130, 131, 175).

Concluding Remarks. A main message of the present study is that there are distinct differences among species with regard to certain transmitter systems and that results in rodent models cannot always be translated directly to humans. Such species differences may be particularly common when studying the coexistence of various transmitters, particularly those related to neuropeptidergic systems (176), although here we show that species differences also may affect NOergic and perhaps glutamatergic systems. However, in general, peptidergic systems are highly conserved. For example, in a comprehensive ISH study, Krolewski, et al. (177) reported that several important neuropeptides are distributed similarly in the human and rodent hypothalamus. Nevertheless, species variations should be taken into account when developing drugs for human disorders. The present evidence for GalR3 signaling in NA-LC and, presumably, in 5-HT-DRN neurons indicates that a similar mechanism may operate in both types of neurons in humans and that GalR3 is a relevant target for drugs aiming to treat humans suffering from anxiety and/or depression. However, to our knowledge, no clinical trials have been carried out with the GalR3 antagonists mentioned above.

Materials and Methods

Subjects. Postmortem frozen brain tissues including LC and DRN from 12 patients were obtained by autopsy at different departments of Semmelweis University (Table S2). Written informed consent was obtained, and studies were approved by the Ethics Committee of Semmelweis University. The postmortem time for the frozen brains, including LC and DRN, was up to 10 h (Table S2).

RNA Probe Synthesis. RNA probes specific to TH, galanin, GalR1, and GalR3 were prepared from human dorsal root ganglion (DRG) mRNA (Clontech). TPH2, GalR2, VGlut 1, VGlut2, VGlut3, and nNOS were generated from human total-brain RNA (Ambion). The human DRG mRNA and total-brain RNA were reverse transcribed to generate cDNA using the Retroscript Kit (Ambion). This cDNA then was amplified using specific primers (Table S3), subcloned into a PCR1II-TOPO vector (Invitrogen), and confirmed by nucleotide sequencing (KIGene). The plasmids were linearized and then transcribed using T7 and SP6 RNA polymerases to generate sense and antisense RNA probes. In vitro transcription was carried out using the MAXIscript SP6/T7 kit (Ambion) and [α^{35}]-UTP (Perkin-Elmer) according to the manufacturer's instructions. The

transcripts then were purified using NucAway Spin Columns (Ambion). Sense probes were used as negative controls.

ISH. Sections (14 μm) for the postmortem human brains were prepared in a cryostat. Fixation, prehybridization, and hybridization were performed as described in *SI Materials and Methods*. Sections were placed against a film and/or dipped in an autoradiographic emulsion, developed, and mounted with glycerol/PBS medium (*SI Materials and Methods*). Sections also were counterstained for cresyl violet (Merck) (*SI Materials and Methods*).

qPCR. Tissue for qPCR analysis was collected as stated in *SI Materials and Methods*. Total RNA was isolated with the Qiagen RNeasy Mini Kit (Qiagen). First-strand cDNA was synthesized and subjected to PCR (*SI Materials and Methods*).

Microscopic Analysis. Sections were analyzed using a Nikon Eclipse E600 microscope equipped with a bright- and dark-field condenser and epi-polarization with side entrance illumination (Fiberoptic-Heim AG) and epi-fluorescence with appropriate filters combinations connected to a digital camera (Nikon DXM 1200). In some cases, Kodak T-MAX 400 black-and-white film was used for photography. Sections were scanned using a Nikon LS-2000 film scanner (Nikon). Scanned and digital images were imported into Adobe PhotoShop 6.0 (Adobe Systems, Inc.) and optimized for brightness, contrast, and sharpness. The atlases of Paxinos and Xu-Feng (178) and Olszewski and Baxter (179) were consulted throughout this work.

ACKNOWLEDGMENTS. We thank Professor Sandra Ceccatelli, Dr. Christina Bark and Dr. Roshan Tofighi for advice with regard to qPCR analysis and Professor Nenad Bogdanovic for valuable advice concerning the neuroanatomy of the human brain stem and Dr. Csaba Adori, Blanca-Silva Lopez, Yu Qian, and Mingdong Zhang for their valuable assistance. This study was supported by the Swedish Research Council (04X-2887); the Marianne and Marcus Wallenberg Foundation; the Knut and Alice Wallenberg Foundation; Grant NEWMOOD; LHSM-CT-2003-503474 from the European Union; the National Alliance for Research on Schizophrenia and Depression; a grant from AFA (the Swedish Insurance company); funds from Karolinska Institutet; and the Swedish Brain Foundation. The studies would not have been possible without the earlier support of an Unrestricted Bristol-Myers-Squibb Neuroscience grant.

1. Falck B, Hillarp N-Å, Thieme G, Torp A (1962) Fluoresence of catecholamines and related compounds with formaldehyde. *J Histochem Cytochem* 10:348–334.
2. Dahlström A, Fuxe K (1964) Evidence for the existence of monoamine neurons in the central nervous system. I. Demonstration of monoamines in the cell bodies of brainstem neurons. *Acta Physiol Scand* 62(Suppl. 232):1–55.
3. Descarries L, Saucier G (1972) Disappearance of the locus coeruleus in the rat after intraventricular 6-hydroxdopamine. *Brain Res* 37(2):310–316.
4. Goldman G, Coleman PD (1981) Neuron numbers in locus coeruleus do not change with age in Fisher 344 rat. *Neurobiol Aging* 2(1):33–36.
5. Aston-Jones G (2004) Locus coeruleus A5 and A7 noradrenergic cell groups. *The Rat Nervous System*, ed Paxinos G (Elsevier, Amsterdam), 3rd Ed, pp 259–294.
6. Moore RY, Bloom FE (1979) Central catecholamine neuron systems: Anatomy and physiology of the norepinephrine and epinephrine systems. *Annu Rev Neurosci* 2:113–168.
7. Keay KA, Bandler R (2004) Periaqueductal gray. *The Rat Nervous System*, ed Paxinos G (Elsevier, Amsterdam), pp 243–257.
8. Steinbusch HW (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience* 6(4):557–618.
9. Fuxe K, Hökfelt T, Ungerstedt U (1970) Central monoaminergic tracts. *Principles of Psychopharmacology*, eds Clark WG, del Gindice J, (Academic Press, London) pp 87–96.
10. Azmitia EC, Gannon PJ (1986) The primate serotonergic system: A review of human and animal studies and a report on Macaca fascicularis. *Adv Neurol* 43:407–468.
11. Törk I, Hornung JP (1990) Raphe nuclei and the serotonergic system. *The Human Nervous System*, ed Paxinos G (Academic, San Diego), pp 1001–1022.
12. Ungerstedt U (1971) Stereotaxic mapping of the monoamine pathways in the rat brain. *Acta Physiol Scand Suppl* 367:1–48.
13. Steinbusch HWM (1984) Serotonin-immunoreactive neurons and their projections in the CNS. *Handbook Of Chemical Neuroanatomy. Classical Transmitters And Transmitter Receptors in the CNS*, eds Björklund A, Hökfelt T, Kuhar MJ (Elsevier, Amsterdam), Vol 3, pp 68–140.
14. Baker KG, Törk I, Hornung JP, Halasz P (1989) The human locus coeruleus complex: An immunohistochemical and three dimensional reconstruction study. *Exp Brain Res* 77(2):257–270.
15. Chan-Palay V, Asan E (1989) Quantitation of catecholamine neurons in the locus coeruleus in human brains of normal young and older adults and in depression. *J Comp Neurol* 287(3):357–372.
16. German DC, Walker BS, Manaye K, Smith WK, Woodward DJ, North AJ (1988) The human locus coeruleus: Computer reconstruction of cellular distribution. *J Neurosci* 8:1776–1788.
17. Baker KG, et al. (1991) Distribution, morphology and number of monoamine-synthesizing and substance P-containing neurons in the human dorsal raphe nucleus. *Neuroscience* 42(3):757–775.
18. Daszuta A, Portalier P (1985) Distribution and quantification of 5-HT nerve cell bodies in the nucleus raphe dorsalis area of C57BL and BALBc mice. Relationship between anatomy and biochemistry. *Brain Res* 360(1-2):58–64.
19. Fu W, et al. (2010) Chemical neuroanatomy of the dorsal raphe nucleus and adjacent structures of the mouse brain. *J Comp Neurol* 518(17):3464–3494.
20. Nusbaum MP, Blitz DM, Swensen AM, Wood D, Marder E (2001) The roles of co-transmission in neural network modulation. *Trends Neurosci* 24(3):146–154.
21. Kupfermann I (1991) Functional studies of cotransmission. *Physiol Rev* 71(3):683–732.
22. Burnstock G (1986) Purines and cotransmitters in adrenergic and cholinergic neurones. *Prog Brain Res* 68:193–203.
23. Merighi A (2002) Costorage and coexistence of neuropeptides in the mammalian CNS. *Prog Neurobiol* 66(3):161–190.
24. Gutiérrez R (2009) *Co-existence and Co-Release of Classical Neurotransmitters* (Springer, New York).
25. Hökfelt T, Johansson O, Ljungdahl A, Lundberg JM, Schultzberg M (1980) Peptidergic neurones. *Nature* 284(5756):515–521.
26. Lundberg JM (1996) Pharmacology of cotransmission in the autonomic nervous system: Integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol Rev* 48(1):113–178.
27. Dun NJ, Dun SL, Förstermann U (1994) Nitric oxide synthase immunoreactivity in rat pontine medullary neurons. *Neuroscience* 59(2):429–445.
28. Wotherspoon G, Albert M, Rattray M, Priestley JV (1994) Serotonin and NADPH-diaphorase in the dorsal raphe nucleus of the adult rat. *Neurosci Lett* 173(1-2):31–36.
29. Johnson MD, Ma PM (1993) Localization of NADPH diaphorase activity in monoaminergic neurons of the rat brain. *J Comp Neurol* 332(4):391–406.
30. Wang QP, Guan JL, Nakai Y (1995) Distribution and synaptic relations of NOS neurons in the dorsal raphe nucleus: A comparison to 5-HT neurons. *Brain Res Bull* 37 (2):177–187.
31. Vincent SR, Kimura H (1992) Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* 46(4):755–784.
32. Xu ZQ, Hökfelt T (1997) Expression of galanin and nitric oxide synthase in subpopulations of serotonin neurons of the rat dorsal raphe nucleus. *J Chem Neuroanat* 13(3):169–187.
33. Gras C, et al. (2002) A third vesicular glutamate transporter expressed by cholinergic and serotoninergic neurons. *J Neurosci* 22(13):5442–5451.
34. Hioki H, et al. (2010) Vesicular glutamate transporter 3-expressing nonserotonergic projection neurons constitute a subregion in the rat midbrain raphe nuclei. *J Comp Neurol* 518(5):668–686.
35. Schäfer MK, Varoqui H, Defamie N, Weihe E, Erickson JD (2002) Molecular cloning and functional identification of mouse vesicular glutamate transporter 3 and its expression in subsets of novel excitatory neurons. *J Biol Chem* 277(52):50734–50748.
36. Amilhon B, et al. (2010) VGLUT3 (vesicular glutamate transporter type 3) contribution to the regulation of serotonergic transmission and anxiety. *J Neurosci* 30(6):2198–2210.
37. Calizo LH, et al. (2011) Raphe serotonin neurons are not homogenous: Electrophysiological, morphological and neurochemical evidence. *Neuropharmacology* 61(3):524–543.
38. Jackson J, Bland BH, Antle MC (2009) Nonserotonergic projection neurons in the midbrain raphe nuclei contain the vesicular glutamate transporter VGLUT3. *Synapse* 63(1):31–41.
39. Lein ES, et al. (2007) Genome-wide atlas of gene expression in the adult mouse brain. *Nature* 445(7124):168–176.
40. Melander T, et al. (1986) Coexistence of galanin-like immunoreactivity with catecholamines, 5-hydroxytryptamine, GABA and neuropeptides in the rat CNS. *J Neurosci* 6(12):3640–3654.
41. Tatemoto K, Rökaeus A, Jörnvall H, McDonald TJ, Mutt V (1983) Galanin - a novel biologically active peptide from porcine intestine. *FEBS Lett* 164(1):124–128.
42. Bersani M, et al. (1991) Human galanin: Primary structure and identification of two molecular forms. *FEBS Lett* 283(2):189–194.
43. Evans HF, Shine J (1991) Human galanin: Molecular cloning reveals a unique structure. *Endocrinology* 129(3):1682–1684.
44. Schmidt WE, et al. (1991) Isolation and primary structure of pituitary human galanin, a 30-residue nonamidated neuropeptide. *Proc Natl Acad Sci USA* 88(24): 11435–11439.
45. Cheung CC, Hohmann JG, Clifton DK, Steiner RA (2001) Distribution of galanin messenger RNA-expressing cells in murine brain and their regulation by leptin in regions of the hypothalamus. *Neuroscience* 103(2):423–432.
46. Rökaeus A, et al. (1984) A galanin-like peptide in the central nervous system and intestine of the rat. *Neurosci Lett* 47(2):161–166.
47. Skofitsch G, Jacobowitz DM (1986) Quantitative distribution of galanin-like immunoreactivity in the rat central nervous system. *Peptides* 7(4):609–613.
48. Melander T, Hökfelt T, Rökaeus A (1986) Distribution of galaninlike immunoreactivity in the rat central nervous system. *J Comp Neurol* 248(4):475–517.
49. Pérez SE, Wynick D, Steiner RA, Mufson EJ (2001) Distribution of galaninergic immunoreactivity in the brain of the mouse. *J Comp Neurol* 434(2):158–185.
50. Skofitsch G, Jacobowitz DM (1985) Immunohistochemical mapping of galanin-like neurons in the rat central nervous system. *Peptides* 6(3):509–546.
51. Iismaa TP, Shine J (1999) Galanin and galanin receptors. *Results Probl Cell Differ* 26: 257–291.

52. Lang R, Gundlach AL, Kofler B (2007) The galanin peptide family: Receptor pharmacology, pleiotropic biological actions, and implications in health and disease. *Pharmacol Ther* 115(2):177–207.
53. Branchek TA, Smith KE, Gerald C, Walker MW (2000) Galanin receptor subtypes. *Trends Pharmacol Sci* 21(3):109–117.
54. Wang S, Gustafson EL (1998) Galanin receptor subtypes. *Drug News Perspect* 11(8): 458–468.
55. Habert-Ortoli E, Amiranoff B, Loquet I, Laburthe M, Mayaux JF (1994) Molecular cloning of a functional human galanin receptor. *Proc Natl Acad Sci USA* 91(21): 9780–9783.
56. Burazin TC, Larm JA, Ryan MC, Gundlach AL (2000) Galanin-R1 and -R2 receptor mRNA expression during the development of rat brain suggests differential subtype involvement in synaptic transmission and plasticity. *Eur J Neurosci* 12(8):2901–2917.
57. Mennicken F, Hoffert C, Pelletier M, Ahmad S, O'Donnell D (2002) Restricted distribution of galanin receptor 3 (GalR3) mRNA in the adult rat central nervous system. *J Chem Neuroanat* 24(4):257–268.
58. O'Donnell D, Ahmad S, Wahlestedt C, Walker P (1999) Expression of the novel galanin receptor subtype GALR2 in the adult rat CNS: Distinct distribution from GALR1. *J Comp Neurol* 409(3):469–481.
59. O'Donnell D, et al. (2003) Localization of galanin receptor subtypes in the rat CNS. *Handbook of Chemical Neuroanatomy. Peptide Receptors, Part II*, eds Quirion R, Björklund A, Hökfelt T (Elsevier, Amsterdam), Vol 20, pp 195–244.
60. Waters SM, Krause JE (2000) Distribution of galanin-1, -2 and -3 receptor messenger RNAs in central and peripheral rat tissues. *Neuroscience* 95(1):265–271.
61. Xu ZQ, Shi TJ, Hökfelt T (1998) Galanin/GMAP- and NPY-like immunoreactivities in locus coeruleus and noradrenergic nerve terminals in the hippocampal formation and cortex with notes on the galanin-R1 and -R2 receptors. *J Comp Neurol* 392(2): 227–251.
62. Holets VR, Hökfelt T, Rökaeus A, Terenius L, Goldstein M (1988) Locus coeruleus neurons in the rat containing neuropeptide Y, tyrosine hydroxylase or galanin and their efferent projections to the spinal cord, cerebral cortex and hypothalamus. *Neuroscience* 24(3):893–906.
63. Chan-Palay V, Jentsch B, Lang W, Hochli M, Asan E (1990) Distribution of neuropeptide Y, C-terminal flanking peptide of NPY and galanin coexistence with catecholamine in the locus coeruleus of normal human, Alzheimer's dementia and Parkinson's disease brains. *Dementia* 1:18–31.
64. Kordower JH, Le HK, Mufson EJ (1992) Galanin immunoreactivity in the primate central nervous system. *J Comp Neurol* 319(4):479–500.
65. Miller MA, Kolb PE, Leverenz JB, Peskind ER, Raskind MA (1999) Preservation of noradrenergic neurons in the locus ceruleus that coexpress galanin mRNA in Alzheimer's disease. *J Neurochem* 73(5):2028–2036.
66. Larm JA, Shen PJ, Gundlach AL (2003) Differential galanin receptor-1 and galanin expression by 5-HT neurons in dorsal raphé nucleus of rat and mouse: Evidence for species-dependent modulation of serotonin transmission. *Eur J Neurosci* 17(3): 481–493.
67. Skofitsch G, Sills MA, Jacobowitz DM (1986) Autoradiographic distribution of 125I-galanin binding sites in the rat central nervous system. *Peptides* 7(6):1029–1042.
68. Pieribone VA, et al. (1995) Galanin induces a hyperpolarization of norepinephrine-containing locus coeruleus neurons in the brainstem slice. *Neuroscience* 64(4): 861–874.
69. Melander T, et al. (1988) Autoradiographic quantitation and anatomical mapping of 125I-galanin binding sites in the rat central nervous system. *J Chem Neuroanat* 1(4): 213–233.
70. Köhler C, Hallman H, Melander T, Hökfelt T, Norheim E (1989) Autoradiographic mapping of galanin receptors in the monkey brain. *J Chem Neuroanat* 2(5):269–284.
71. Köhler C, et al. (1989) Distribution of galanin-binding sites in the monkey and human telencephalon: Preliminary observations. *Exp Brain Res* 75(2):375–380.
72. Köhler C, Chan-Palay V (1990) Galanin receptors in the post-mortem human brain. Regional distribution of 125I-galanin binding sites using the method of in vitro receptor autoradiography. *Neurosci Lett* 120(2):179–182.
73. Xu ZQ, Zhang X, Pieribone VA, Grillner S, Hökfelt T (1998) Galanin-5-hydroxytryptamine interactions: Electrophysiological, immunohistochemical and in situ hybridization studies on rat dorsal raphe neurons with a note on galanin R1 and R2 receptors. *Neuroscience* 87(1):79–94.
74. Nagatsu T, Levitt M, Udenfriend S (1964) Tyrosine Hydroxylase. The Initial Step in Norepinephrine Biosynthesis. *J Biol Chem* 239:2910–2917.
75. Grima B, et al. (1987) A single human gene encoding multiple tyrosine hydroxylases with different predicted functional characteristics. *Nature* 326(6114):707–711.
76. Walther DJ, et al. (2003) Synthesis of serotonin by a second tryptophan hydroxylase isoform. *Science* 299(5603):76.
77. Bredt DS, et al. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* 351(6329):714–718.
78. Geller DA, et al. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc Natl Acad Sci USA* 90(8):3491–3495.
79. Fremeau RT, Jr., Voglmaier S, Seal RP, Edwards RH (2004) VGLUTs define subsets of excitatory neurons and suggest novel roles for glutamate. *Trends Neurosci* 27(2): 98–103.
80. Kaneko T, Fujiyama F (2002) Complementary distribution of vesicular glutamate transporters in the central nervous system. *Neurosci Res* 42(4):243–250.
81. Masson J, Sagné C, Hamon M, El Mestikawy S (1999) Neurotransmitter transporters in the central nervous system. *Pharmacol Rev* 51(3):439–464.
82. Le Maitre E, Diaz-Heijtz R, Palkovits M, Hökfelt T (2008) The galanin system in the human locus coeruleus and dorsal raphe nucleus. *J Neural Transm* 11:1724–1172.
83. Le Maitre E, Diaz-Heijtz R, Palkovits M, Hökfelt T (2009) The galanin system in the human brain and relations to serotonin and noradrenaline neurons. *Eur Neuropsychopharmacol* 19:S18.
84. Ma X, et al. (2001) Effects of galanin receptor agonists on locus coeruleus neurons. *Brain Res* 919(1):169–174.
85. Smith KE, et al. (1998) Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K+ channels. *J Biol Chem* 273(36):23321–23326.
86. Wang S, He C, Hashemi T, Bayne M (1997) Cloning and expressional characterization of a novel galanin receptor. Identification of different pharmacophores within galanin for the three galanin receptor subtypes. *J Biol Chem* 272(51):31949–31952.
87. Whitehouse PJ, et al. (1982) Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain. *Science* 215(4537):1237–1239.
88. Melander T, et al. (1985) Galanin-like immunoreactivity in cholinergic neurons of the septum-basal forebrain complex projecting to the hippocampus of the rat. *Brain Res* 360(1-2):130–138.
89. Senut MC, Menetrey D, Lamour Y (1989) Cholinergic and peptidergic projections from the medial septum and the nucleus of the diagonal band of Broca to dorsal hippocampus, cingulate cortex and olfactory bulb: A combined wheatgerm agglutinin-apohorseradish peroxidase-gold immunohistochemical study. *Neuroscience* 30(2): 385–403.
90. Cortés R, Ceccatelli S, Schalling M, Hökfelt T (1990) Differential effects of intracerebroventricular colchicine administration on the expression of mRNAs for neuropeptides and neurotransmitter enzymes, with special emphasis on galanin: An in situ hybridization study. *Synapse* 6(4):369–391.
91. Agoston DV, Komoly S, Palkovits M (1994) Selective up-regulation of neuropeptide synthesis by blocking the neuronal activity: Galanin expression in septohippocampal neurons. *Exp Neurol* 126(2):247–255.
92. Palkovits M (1995) Neuropeptide messenger plasticity in the CNS neurons following axotomy. *Mol Neurobiol* 10(2-3):91–103.
93. Miller MA, Kolb PE, Planas B, Raskind MA (1998) Few cholinergic neurons in the rat basal forebrain coexpress galanin messenger RNA. *J Comp Neurol* 391(2):248–258.
94. Melander T, Staines WA (1986) A galanin-like peptide coexists in putative cholinergic somata of the septum-basal forebrain complex and in acetylcholinesterase-containing fibers and varicosities within the hippocampus in the owl monkey (Aotus trivirgatus). *Neurosci Lett* 68(1):17–22.
95. Kordower JH, Mufson EJ (1990) Galanin-like immunoreactivity within the primate basal forebrain: Differential staining patterns between humans and monkeys. *J Comp Neurol* 294(2):281–292.
96. Chan-Palay V, Köhler C, Ernfors P, Persson H (1991) Senile dementia of the Alzheimer type is accompanied by hypertrophy of galanin axons, reservation of receptors and tissue specific expression of the galanin gene in the nucleus basalis Meynert. *Galanin. A New Multifunctional Peptide in the Neuro-Endocrine System*, eds Hökfelt T, Bartfai T, Jacobowitz D, Ottoson D (MacMillan, London), pp 419–427.
97. Counts SE, Perez SE, Ginsberg SD, Mufson EJ (2010) Neuroprotective role for galanin in Alzheimer's disease. *EXS* 102:143–162.
98. Chotard C, et al. (2002) Effects of histamine H3 receptor agonist and antagonist on histamine co-transmitter expression in rat brain. *J Neural Transm* 109(3):293–306.
99. Köhler C, et al. (1986) Galanin immunoreactivity in hypothalamic neurons: Further evidence for multiple chemical messengers in the tuberomammillary nucleus. *J Comp Neurol* 250(1):58–64.
100. Lantos TA, Görcs TJ, Palkovits M (1995) Immunohistochemical mapping of neuropeptides in the premamillary region of the hypothalamus in rats. *Brain Res Brain Res Rev* 20(2):209–249.
101. Trottier S, et al. (2002) Co-localization of histamine with GABA but not with galanin in the human tuberomamillary nucleus. *Brain Res* 939(1-2):52–64.
102. Carrive P, Morgan MM (2004) Periaqueductal gray. *The Human Nervous System*, eds Paxinos G, Mai JK (Elsevier, San Diego), 2nd Ed.
103. Hope BT, Michael GJ, Knigge KM, Vincent SR (1991) Neuronal NADPH diaphorase is a nitric oxide synthase. *Proc Natl Acad Sci USA* 88(7):2811–2814.
104. Bredt DS, et al. (1991) Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. *Neuron* 7(4):615–624.
105. Oliveira AL, et al. (2003) Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia. *Synapse* 50(2):117–129.
106. Brumovsky PR, et al. (2011) Expression of vesicular glutamate transporters type 1 and 2 in sensory and autonomic neurons innervating the mouse colorectum. *J Comp Neurol* 519(16):3346–3366.
107. Mintz EM, Scott TJ (2006) Colocalization of serotonin and vesicular glutamate transporter 3-like immunoreactivity in the midbrain raphe of Syrian hamsters (Mesocricetus auratus). *Neurosci Lett* 394(2):97–100.
108. Anonymous. Allen Brain Atlas Resources (Allen Institute for Brain Research, Seattle). Available at: www.brain-map.org.
109. Anonymous. Allen Human Cortex Study (Allen Institute for Brain Research, Seattle). Available at http://humancortex.alleninstitute.org.
110. Rhodes KJ, Trimmer JS (2006) Antibodies as valuable neuroscience research tools versus reagents of mass distraction. *J Neurosci* 26(31):8017–8020.
111. Saper CB (2005) An open letter to our readers on the use of antibodies. *J Comp Neurol* 493(4):477–478.
112. Bartfai T, Buckley PT, Eberwine J (2012) Drug targets: Single-cell transcriptomics hastens unbiased discovery. *Trends Pharmacol Sci* 33(1):9–16.
113. Eberwine J, Bartfai T (2011) Single cell transcriptomics of hypothalamic warm sensitive neurons that control core body temperature and fever response Signaling

asymmetry and an extension of chemical neuroanatomy. *Pharmacol Ther* 129(3): 241–259.
114. Tofighi R, et al. (2012) Galanin and its three receptors in human pituitary adenoma. *Neuropeptides* 46(5):195–201.
115. Svensson TH, Bunney BS, Aghajanian GK (1975) Inhibition of both noradrenergic and serotonergic neurons in brain by the alpha-adrenergic agonist clonidine. *Brain Res* 92(2):291–306.
116. Aghajanian GK, Cedarbaum JM, Wang RY (1977) Evidence for norepinephrine-mediated collateral inhibition of locus coeruleus neurons. *Brain Res* 136(3):570–577.
117. Cedarbaum JM, Aghajanian GK (1977) Catecholamine receptors on locus coeruleus neurons: Pharmacological characterization. *Eur J Pharmacol* 44(4):375–385.
118. Aghajanian GK, Wang RY (1978) Physiology and pharmacology of central serotonergic neurons. *Psychopharmacology: A Generation of Progress*, eds Lipton MA, DiMasco A, Killam KF (Elsevier, Amsterdam).
119. Ludwig M (2005) *Dendritic Neurotransmitter Release* (Springer, New York).
120. Huang HP, et al. (2007) Long latency of evoked quantal transmitter release from somata of locus coeruleus neurons in rat pontine slices. *Proc Natl Acad Sci USA* 104 (4):1401–1406.
121. Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci* 7(2):126–136.
122. Vila-Porcile E, et al. (2009) Dendritic synthesis and release of the neuropeptide galanin: Morphological evidence from studies on rat locus coeruleus neurons. *J Comp Neurol* 516(3):199–212.
123. Artigas F, Romero L, de Montigny C, Blier P (1996) Acceleration of the effect of selected antidepressant drugs in major depression by 5-HT1A antagonists. *Trends Neurosci* 19(9):378–383.
124. Millan MJ (2006) Multi-target strategies for the improved treatment of depressive states: Conceptual foundations and neuronal substrates, drug discovery and therapeutic application. *Pharmacol Ther* 110(2):135–370.
125. Seutin V, Verbanck P, Massotte L, Dresse A (1989) Galanin decreases the activity of locus coeruleus neurons in vitro. *Eur J Pharmacol* 164(2):373–376.
126. Sevcik J, Finta EP, Illes P (1993) Galanin receptors inhibit the spontaneous firing of locus coeruleus neurones and interact with mu-opioid receptors. *Eur J Pharmacol* 230(2):223–230.
127. Xu ZQ, Tong YG, Hökfelt T (2001) Galanin enhances noradrenaline-induced outward current on locus coeruleus noradrenergic neurons. *Neuroreport* 12(8):1779–1782.
128. Hawes JJ, Brunzell DH, Wynick D, Zachariou V, Picciotto MR (2005) GalR1, but not GalR2 or GalR3, levels are regulated by galanin signaling in the locus coeruleus through a cyclic AMP-dependent mechanism. *J Neurochem* 93(5):1168–1176.
129. Mitsukawa K, Lu X, Bartfai T (2009) Bidirectional regulation of stress responses by galanin in mice: Involvement of galanin receptor subtype 1. *Neuroscience* 160(4): 837–846.
130. Swanson CJ, et al. (2005) Anxiolytic- and antidepressant-like profiles of the galanin-3 receptor (Gal3) antagonists SNAP 37889 and SNAP 398299. *Proc Natl Acad Sci USA* 102(48):17489–17494.
131. Barr AM, et al. (2006) A novel, systemically active, selective galanin receptor type-3 ligand exhibits antidepressant-like activity in preclinical tests. *Neurosci Lett* 405(1-2): 111–115.
132. Sharkey LM, Madamba SG, Siggins GR, Bartfai T (2008) Galanin alters GABAergic neurotransmission in the dorsal raphe nucleus. *Neurochem Res* 33(2):285–291.
133. Borroto-Escuela DO, et al. (2010) Galanin receptor-1 modulates 5-hydroxtryptamine-1A signaling via heterodimerization. *Biochem Biophys Res Commun* 393(4):767–772.
134. Fuxe K, et al. (2008) Receptor-receptor interactions within receptor mosaics. Impact on neuropsychopharmacology. *Brain Res Brain Res Rev* 58(2):415–452.
135. Maes M, Meltzer HY (1995) The serotonin hypothesis of major depression. *Psychopharmacology: The Fourth Generation of Progress*, eds Bloom FE, Kupfer DJ (Raven, New York), pp 933–944.
136. Montgomery SA (2006) Why do we need new and better antidepressants? *Clin Psychopharmacol* 21(Suppl 1):S1–S10.
137. Schatzberg AF, Schildkrant JJ (1995) Recent studies on norepinephrine systems in mood disorders. *Pharmacology: The Fourth Generation of Progress*, eds Bloom FE, Kupfer DJ (Raven, New York), pp 933–944.
138. Maubach KA, Rupniak NM, Kramer MS, Hill RG (1999) Novel strategies for pharmacotherapy of depression. *Curr Opin Chem Biol* 3(4):481–488.
139. Nemeroff CB, Vale WW (2005) The neurobiology of depression: Inroads to treatment and new drug discovery. *J Clin Psychiatry* 66(Suppl 7):5–13.
140. Reul JM, Holsboer F (2002) Corticotropin-releasing factor receptors 1 and 2 in anxiety and depression. *Curr Opin Pharmacol* 2(1):23–33.
141. Sajdyk TJ, Shekhar A, Gehlert DR (2004) Interactions between NPY and CRF in the amygdala to regulate emotionality. *Neuropeptides* 38(4):225–234.
142. Holmes A, Heilig M, Rupniak NM, Steckler T, Griebel G (2003) Neuropeptide systems as novel therapeutic targets for depression and anxiety disorders. *Trends Pharmacol Sci* 24(11):580–588.
143. Nestler EJ, Carlezon WA, Jr. (2006) The mesolimbic dopamine reward circuit in depression. *Biol Psychiatry* 59(12):1151–1159.
144. Weiss JM, Bonsall RW, Demetrikopoulos MK, Emery MS, West CHK (1998) Galanin: A significant role in depression? *Ann N Y Acad Sci* 863:364–382.
145. Fuxe K, et al. (1991) Galanin/5-HT interactions in the rat central nervous system. Relevance for depression. *Galanin: A New Multifunctional Peptide in the Neuro-Endocrine System. Wenner-Gren Center International Symposium Series, Vol.* 58, eds Hökfelt T, Bartfai T, Jacobowitz D, Ottoson D (MacMillan, London), pp 221–235.
146. Barrera G, et al. (2005) One for all or one for one: Does co-transmission unify the concept of a brain galanin "system" or clarify any consistent role in anxiety? *Neuropeptides* 39(3):289–292.
147. Ögren SO, Kuteeva E, Hökfelt T, Kehr J (2006) Galanin receptor antagonists : A potential novel pharmacological treatment for mood disorders. *CNS Drugs* 20(8): 633–654.
148. Lu X, Sharkey L, Bartfai T (2007) The brain galanin receptors: Targets for novel antidepressant drugs. *CNS Neurol Disord Drug Targets* 6(3):183–192.
149. Bing O, Möller C, Engel JA, Söderpalm B, Heilig M (1993) Anxiolytic-like action of centrally administered galanin. *Neurosci Lett* 164(1-2):17–20.
150. Möller C, Sommer W, Thorsell A, Heilig M (1999) Anxiogenic-like action of galanin after intra-amygdala administration in the rat. *Neuropsychopharmacology* 21(4): 507–512.
151. Kuteeva E, Hökfelt T, Wardi T, Ögren S-O (2010) Galanin, galanin receptor subtypes and depression-like behaviour. *EXS* 102:163–181.
152. Belfer I, et al. (2007) Alcoholism is associated with GALR3 but not two other galanin receptor genes. *Genes Brain Behav* 6(5):473–481.
153. Belfer I, et al. (2006) Association of galanin haplotypes with alcoholism and anxiety in two ethnically distinct populations. *Mol Psychiatry* 11(3):301–311.
154. Levran O, et al. (2008) Genetic susceptibility to heroin addiction: A candidate gene association study. *Genes Brain Behav* 7(7):720–729.
155. Unschuld PG, et al. (2008) Polymorphisms in the galanin gene are associated with symptom-severity in female patients suffering from panic disorder. *J Affect Disord* 105(1-3):177–184.
156. Unschuld PG, et al. (2010) Gender-specific association of galanin polymorphisms with HPA-axis dysregulation, symptom severity, and antidepressant treatment response. *Neuropsychopharmacology* 35(7):1583–1592.
157. Davidson S, et al. (2011) Differential activity by polymorphic variants of a remote enhancer that supports galanin expression in the hypothalamus and amygdala: Implications for obesity, depression and alcoholism. *Neuropsychopharmacology* 36 (11):2211–2221.
158. Wray NR, et al. (2012) Genome-wide association study of major depressive disorder: New results, meta-analysis, and lessons learned. *Mol Psychiatry* 17(1):36–48.
159. Jackson KJ, Chen X, Miles MF, Harenza J, Damaj MI (2011) The neuropeptide galanin and variants in the GalR1 gene are associated with nicotine dependence. *Neuropsychopharmacology* 36(11):2339–2348.
160. Lori A, et al. (2011) The galanin receptor 1 gene associates with tobacco craving in smokers seeking cessation treatment. *Neuropsychopharmacology* 36(7):1412–1420.
161. Svensson TH (1987) Stress, central neurotransmitters, and the mechanism of action of alpha 2-adrenoceptor agonists. *J Cardiovasc Pharmacol* 10(Suppl 12):S88–S92.
162. Page ME, Valentino RJ (1994) Locus coeruleus activation by physiological challenges. *Brain Res Bull* 35(5-6):557–560.
163. Aston-Jones G, Rajkowski J, Kubiak P, Valentino RJ, Shipley MT (1996) Role of the locus coeruleus in emotional activation. *Prog Brain Res* 107:379–402.
164. Weiss JM, et al. (1994) Depression and anxiety: Role of the locus coeruleus and corticotropin-releasing factor. *Brain Res Bull* 35(5-6):561–572.
165. Bremner JD, Krystal JH, Southwick SM, Charney DS (1996) Noradrenergic mechanisms in stress and anxiety: I. Preclinical studies. *Synapse* 23(1):28–38.
166. Bremner JD, Krystal JH, Southwick SM, Charney DS (1996) Noradrenergic mechanisms in stress and anxiety: II. Clinical studies. *Synapse* 23(1):39–51.
167. Harro J, Oreland L (2001) Depression as a spreading adjustment disorder of monoaminergic neurons: A case for primary implication of the locus coeruleus. *Brain Res Brain Res Rev* 38(1-2):79–128.
168. Charney DS (2004) Psychobiological mechanisms of resilience and vulnerability: Implications for successful adaptation to extreme stress. *Am J Psychiatry* 161(2): 195–216.
169. Foote SL, Bloom FE, Aston-Jones G (1983) Nucleus locus ceruleus: New evidence of anatomical and physiological specificity. *Physiol Rev* 63(3):844–914.
170. Holmes PV, Blanchard DC, Blanchard RJ, Brady LS, Crawley JN (1995) Chronic social stress increases levels of preprogalanin mRNA in the rat locus coeruleus. *Pharmacol Biochem Behav* 50(4):655–660.
171. Sweerts BW, Jarrott B, Lawrence AJ (1999) Expression of preprogalanin mRNA following acute and chronic restraint stress in brains of normotensive and hypertensive rats. *Brain Res Mol Brain Res* 69(1):113–123.
172. Murck H, et al. (1999) Galanin has REM-sleep deprivation-like effects on the sleep EEG in healthy young men. *J Psychiatr Res* 33(3):225–232.
173. Murck H, et al. (2004) Intravenous administration of the neuropeptide galanin has fast antidepressant efficacy and affects the sleep EEG. *Psychoneuroendocrinology* 29 (9):1205–1211.
174. Mitsukawa K, Lu X, Bartfai T (2008) Galanin, galanin receptors and drug targets. *Cell Mol Life Sci* 65(12):1796–1805.
175. Konkel MJ, et al. (2006) 3-arylimino-2-indolones are potent and selective galanin GAL3 receptor antagonists. *J Med Chem* 49(13):3757–3758.
176. Bowers CW (1994) Superfluous neurotransmitters? *Trends Neurosci* 17(8):315–320.
177. Krolewski DM, et al. (2010) Expression patterns of corticotropin-releasing factor, arginine vasopressin, histidine decarboxylase, melanin-concentrating hormone, and orexin genes in the human hypothalamus. *J Comp Neurol* 518(22):4591–4611.
178. Paxinos G, Huang X-F (1995) *Atlas of the Human Brain Stem* (Academic, San Diego).
179. Olszewski J, Baxter D (1982) *Cytoarchitecture of the Human Brain Stem* (Karger, Basel).