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Steroid Levels in Tadpole (*Rana catesbeiana*) Brain at the Loss and Return of the Righting Response

WAI MAN MOK AND NEIL REESE KRIEGER[1]

Department of Anesthesia, Harvard Medical School, Brigham & Women's Hospital, Boston, MA 02115

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MOK, W. M. AND N. R. KRIEGER. *Steroid levels in tadpole (*Rana catesbeiana*) brain at the loss and return of the righting response.* PHARMACOL BIOCHEM BEHAV 43(2) 523-528, 1992.—Steroid levels were determined at the onset of anesthesia (tLRR) and at the recovery from anesthesia (tRRR). When 3α-hydroxy-5α-pregnan-20-one (3α) was the administered agent, 3α levels were similar at tLRR and $tRRR_1$. The net rate of uptake of 3α by the brain was approximately eight times the rate of loss of 3α. Levels of 3α were twice as high at tLRR when progesterone (PG) was the administered agent as when 3α was used. The presence and absence of 0.1% ethanol in the incubation bath had no detectable effect on steroid levels. A model for the actions of 3α is described. It is argued that interactions between 3α and the target site are specific with respect to chemical structure and that the amount of 3α in the compartment that contains the target sites is always small compared to that in whole brain.

Steroid Pregnanolone 3α-Hydroxy-5α-pregnan-20-one Progesterone Brain Anesthetic General anesthesia

MODELS for the actions of anesthetics are difficult to formulate because anesthetic levels at the target sites and the locale of the target sites have not been determined. It is not known whether anesthetic agents bind diffusely at sites throughout the brain or act at restricted sites (4,11,15,16). Moreover, for steroids metabolite formation in brain makes it difficult to identify the active agent. In this study, a model is presented that relates anesthetic behavior to steroid receptor interactions. Because anesthesia is defined in behavioral terms, behavioral endpoints are used to assess the anesthetized state. These include the time of the loss of the righting response (tLRR) and the time of the return of the righting response (tRRR).

In the tadpole, anesthetic steroids are taken up from an aqueous reservoir via the gill. Anesthesia is reversed by the transfer of the tadpole to a second reservoir that is free of steroid. For relatively soluble steroids such as progesterone (PG), ethanol or other organic solvents are not needed to keep the steroid in solution during incubation. These methods are advantageous over those that are required with a mammal, where a lipid delivery vehicle is needed for injection of anesthetic steroids.

We have recently shown that when PG is the administered anesthetic agent its metabolite, 3α-hydroxy-5α-pregnan-20-one (3α), mediates activity (8) and also that when 3α is the administered agent it is the active molecular species (6). Here, 3α and PG were separately administered to tadpoles (*Rana catesbeiana*), and their levels and the levels of their metabolites were measured in brain.

METHOD

The methods used here are similar to those described previously for the quantitation of PG and its metabolite levels in tadpole brain and are presented briefly except where the use of [^{3}H]3α involves additional methods (8). Tadpoles (*Rana catesbeiana*; 7 g) at the stage just preceding the sprouting of limbs were obtained from Connecticut Valley Biological Supply (Southampton, MA) and from Carolina Biological Supply Company (Burlington, NC). [^{3}H]3α (97% pure) was synthesized from [^{3}H]PG (55 Ci/mmol; New England Nuclear-DuPont; Boston, MA) according to the method of Mok and Krieger (9). No differences in appearance or brain steroid levels were apparent in tadpoles obtained from the two sources. They were individually incubated in 100 ml vol distilled and deionized water with 0.1% ethanol containing [^{3}H]3α (1.3×10^6 dpm, 24°C). No tadpole was used more than once. Aliquots from the bath were centrifuged to remove suspended material and 3α concentration (3.5 μM) was determined from the measurement of radioactivity. Concentration of 3α decreased slightly (< 10%) during the incubation, probably from uptake into tadpoles.

[1] To whom requests for reprints should be addressed.

Tadpoles were killed by decapitation. The brain was removed, weighed (~20 mg wet weight), homogenized (Teflon glass) on ice in 200 μl ethanol, and centrifuged (36,500 × g; 4°C, 15 min). An aliquot of the supernatant (20 μl) was counted to determine the total steroid level in the brain and the remainder of the supernatant was analyzed by thin-layer chromatography (TLC). The pellet was re-extracted in 250 μl ethanol and then digested in a mixture of 200 μl ethanol and 800 μl protosol (New England Nuclear). Both the second supernatant and the digested pellet were also counted by liquid scintillation spectrometry (LSC). The recoveries during extraction were greater than 98%.

TLC was used to resolve related steroids (Gelman instant thin-layer silica acid plates, Fischer, King of Prussia, PA; and chloroform as the mobile phase). Each channel of the card was cut into 0.5-cm segments, placed in scintillation fluid, and counted by liquid scintillation spectrometry. Recovery from TLC was 97–100%. Unlabeled steroid standards [PG, 5α-pregnanedione (5α), and 3α; Sigma Chemical Co., St. Louis, MO] were run in parallel and visualized with 2% perchloric acid. [^{3}H]3α standards were also run in parallel and detected by LSC. cpm was plotted vs. distance of migration. Five peaks were routinely observed with R_f—0.41, 0.03, 0.13, 0.32, and 0.74—and designated 3α, M_0 M_1, M_2 and M_3, respectively. The identity of 3α was confirmed by rechromatography upon high-performance liquid chromatography (HPLC) (C_{18} μ-bondapak column, 70% methanol). M_3 was tentatively identified as 5α by comparison to authentic 5α upon TLC and HPLC. It is not known whether the conversions to metabolites are occurring centrally or peripherally with transport to the brain. Incubations of tadpole brain homogenates with [^{3}H]3α produced the same four metabolites. Although chemical structures for M_0, M_1, and M_2 were not identified, their production in vitro indicates that enzymes are present in tadpole brain that can convert 3α or 5α to these metabolites.

The area under each peak (above background and corrected for overlap with neighboring peaks) was used to calculate the amount of each steroid in units of pmol as previously described (6). For overlapping peaks, the areas were separated by dropping a perpendicular from the valley between them. The counts per minute at the valley were divided between the two bordering peaks by a ratio related to their heights. Radiospecific activities of metabolites were set equal to 3α. No corrections were made for possible contributions from endogenous steroid levels, which were assumed to be far lower than the levels actually determined in brain. For other species, endogenous brain levels are known to be very low (<0.03 pmol/mg) (2,5,14).

The rate of uptake for 3α (and for PG as well) was defined as the net rate, that is, the total rate of uptake minus the rate of loss from the brain (see Table 1). It was assumed that for a given tadpole the total rate of uptake is constant with time and that the different metabolites leave the brain at similar rates. The rate of accumulation of 3α was defined as the rate of uptake minus the rates of loss and of conversion to its metabolites. It was approximated that all three of these rates remained constant with time, although the rate of metabolite formation, in fact, depends upon the steroid level.

Values for the brain levels and times are expressed as means ± SEM. Student's *t*-test was used to evaluate statistical significance (17). Behavioral endpoints included the loss of the righting response (LRR) and the return of the righting response (RRR) and were assayed according to Mok and Krieger (8). tLRR and tRRR are the times at which each behavior occurs. LRR was said to have occurred when the tadpole failed to right itself for 0.5 min after being placed on its back. Following LRR, tadpoles were transferred from the incubation bath to water without steroid where they remained until they reached RRR. RRR_1 was said to have occurred when, following LRR and the transfer to water free of steroid, the tadpole righted itself spontaneously. This is essentially the definition designated previously as RRR (8). RRR_2 was defined as having occurred when the tadpole righted itself within 10 s after being placed on its back for 8 of 10 trials. At RRR_2, the tadpole was awake and could swim away.

When $tRRR_1$ and $tRRR_2$ were observed to coincide (approximately 30% of trials), the tadpole was assigned to the RRR_2 group. This assignment could only be established with certainty for those cases where both behavioral assays were performed. When the tadpole was killed at $tRRR_1$ to assay steroid levels, $tRRR_2$ could not be determined. In such cases, when $tRRR_1$ occurred at greater than 40 min the tadpole was assigned to the RRR_2 group.

Analgesia was also assessed in this study by pinching the tadpole during the course of incubation with a large tweezer at a point approximately 0.5 cm from the tip of the tail. However, as in our related study (8), no evidence of analgesia was observed and these observations are not discussed further.

RESULTS

LRR

Tadpoles were incubated with [^{3}H]3α and killed at tLRR. Figure 1A shows levels for 3α, its metabolites (5α, M_0, M_1, M_2), and total steroid levels (*T*) at tLRR. With few exceptions, levels were higher among tadpoles that reached LRR at later times. Levels for 3α ranged from 6 pmol/mg brain tissue at the earliest tLRR to 13 pmol/mg at 30 to 40 min.

Figure 1B shows the corresponding brain levels (mean ± SEM; n = 18). Levels of 3α were 10.28 ± 1.07 pmol/mg. This is only 28% of *T*. The net rate of uptake of 3α by the brain under these conditions was calculated to be 1.28 ± 0.06 pmol mg^{-1} min^{-1} (mean ± SEM; n = 18). The net rate of accumulation of 3α was calculated to be 0.36 ± 0.03 pmol mg^{-1} min^{-1}.

Ethanol in the Incubation Bath

Ethanol (0.1%) was routinely present to increase the solubility of 3α. It was essential to establish whether or not it contributed to the steroid levels or to tLRR. To accomplish this, incubations were carried out in which tadpoles were preincubated with 0.1% ethanol for 30 min and then transferred to a second bath that contained both 3α and ethanol. Tadpoles were then incubated in the standard fashion until LRR. Figure 1C shows the brain levels (mean ± SEM; n = 8) for 3α and its metabolites. Steroid levels and the rate of uptake measured under conditions with preincubation did not differ significantly from those obtained in Fig. 1B. The uptake rate for 3α was 1.32 ± 0.07 pmol mg^{-1} min^{-1}.

Levels of 3α were further compared at tLRR in the presence and absence of ethanol by using PG as the precursor of 3α. PG is soluble in water without ethanol. No significant differences were observed for 3α ($p > 0.4$), the agent responsible for anesthetic activity, or for PG or any of its other metabolites, for incubations in the presence or in the absence of ethanol, nor did tLRR occur earlier when ethanol was present (not shown). It was concluded that 0.1% ethanol in the incubation bath had a negligible effect upon steroid brain levels at LRR.

TABLE 1

STEROID LEVELS, RATES OF UPTAKE, AND RATES OF ACCUMULATION (MEAN ± SEM)

Steroid Administered	tLRR (min)	Total Steroids (pmol/mg)	3α (pmol/mg)	Rate of Steroid Uptake (pmol mg^{-1} min^{-1})	Rate of Accumulation of 3α (pmol mg^{-1} min^{-1})
3α*	37.3 ± 3.5	28.6 ± 2.6	6.8 ± 0.9	0.77 ± 0.02	0.18 ± 0.02
PG†	27.9 ± 1.2	107.1 ± 15.3	15.9 ± 1.6	3.87 ± 0.63	0.58 ± 0.06
3α‡	29.3 ± 2.7	36.2 ± 3.2	10.3 ± 1.1	1.28 ± 0.06	0.36 ± 0.03
PG§	25.3 ± 2.9	112.8 ± 13.5	21.8 ± 4.0	4.74 ± 0.78	0.91 ± 0.21

*3α (2.6 μM); n = 3.
†PG (18.2 μM); n = 4.
‡3α (3.5 μM); Fig. 1B, n = 18.
‡PG (20 μM); n = 10 [includes four tadpoles from previous study (6)].

Comparison of 3α Levels at LRR for Anesthesia with 3α and with PG

We had originally supposed that 3α levels at LRR would reflect conditions at the behavioral endpoint and therefore would not differ with different administered agents. Comparison of 3α levels with those previously obtained for 3α derived from PG (Table 1; ‡, §) showed that they did differ. When PG was the administered agent as compared to 3α, 3α levels were approximately two times higher and the rates of uptake for PG were four times faster than for 3α. (The bath concentration of PG was six times higher than that of 3α.) Similarly, the rate of accumulation of 3α was two to three times greater when PG was the administered agent.

The comparison was repeated in parallel under essentially the same experimental conditions as before. Levels of 3α were compared at tLRR for incubations with 3α or with PG (Table 1; *, †). Levels of 3α were again seen to be 100% greater when PG was the administered agent ($p < 0.02$).

RRR

The frequencies of occurrence of RRR_1 (solid) and RRR_2 (open) as functions of time after LRR for a series of 23 tadpoles are shown in Fig. 2A. For 7 of the 23 tadpoles, $tRRR_1$ and $tRRR_2$ coincided; these 7 were grouped and plotted with the RRR_2 group. Seventy-five percent of the tadpoles that reached $tRRR_1$ did so between 0–20 min after LRR and 70% of tadpoles that reached $tRRR_2$ did so between 20–60 min after LRR.

In Fig. 2B, brain steroid levels (mean ± SEM) are shown for tadpoles following killing at RRR_1 (8 ± 2 min; n = 13) and also following killing at RRR_2 (45 ± 8 min; n = 11). At RRR_1, levels for 3α were 9.54 ± 1.02 pmol/mg. Levels for

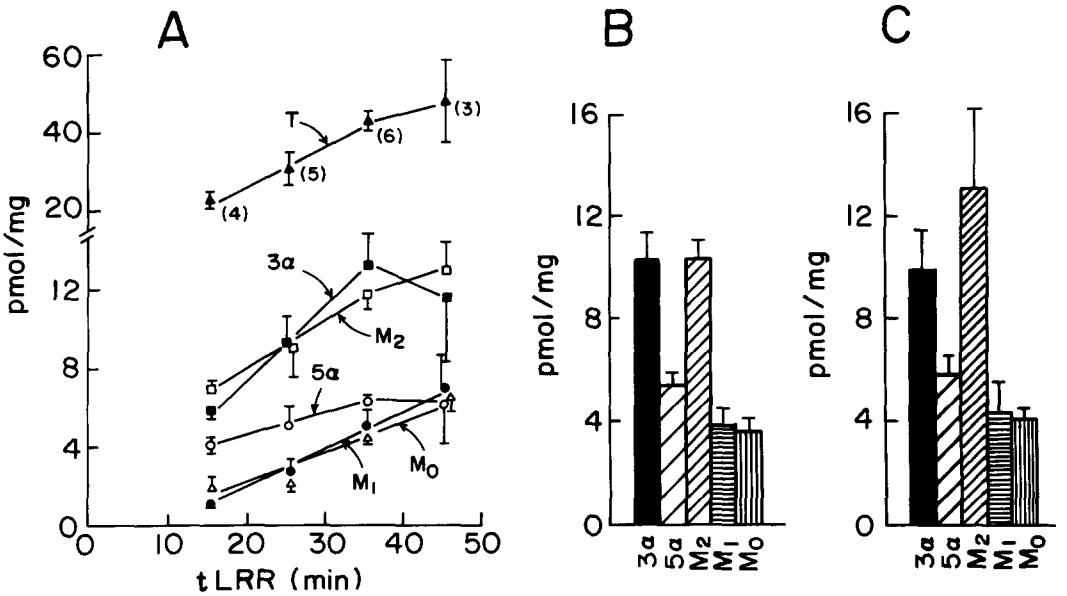

FIG. 1. Steroid brain levels in tadpole brain. (A). Brain levels were quantitated at the time of the loss of the righting response. Values are grouped at the midpoint of each 10-min interval. The number of tadpoles is shown in parentheses. (B). Bar heights correspond to steroid levels at LRR (pmol/mg brain tissue, mean ± SEM; n = 18). Total steroid levels were 36.2 ± 3.15 pmol/mg. (C). Steroid brain levels after preincubation in ethanol. Bar heights indicate brain levels (pmol/mg, mean ± SEM; n = 8) at LRR. Total steroid levels were 40.0 ± 5.7 pmol/mg. 3α, 3α-hydroxy-5α-pregnan-20-one; M_0, M_1, M_2, and 5α are metabolites of 3α. T, total steroids.

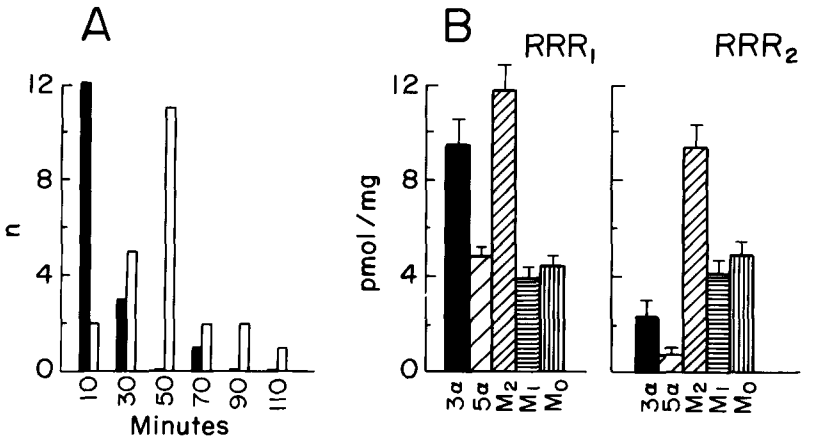

FIG. 2. Behavioral endpoints, RRR_1 and RRR_2. (A). The number of tadpoles (n) that reached the endpoint is plotted for successive 20-min intervals. The bars are drawn at approximately the middle of the interval. (RRR_1, solid, $n = 16$; RRR_2, open, $n = 23$) (B). Steroid brain levels (pmol/mg brain tissue, mean ± SEM). RRR_1, $n = 13$; RRR_2, $n = 11$. 3α, 3α-hydroxy-5α-pregnan-20-one; M_0, M_1, M_2, and 5α are metabolites of 3α. Total steroid levels were 36.9 ± 2.7 and 23.4 ± 2.1 pmol/mg at RRR_1 and RRR_2, respectively.

3α, 5α, M_0, M_1, and M_2 at RRR_1 did not differ significantly from those at LRR (Fig. 1B). At RRR_2, levels for 3α were 2.33 ± 0.71 pmol/mg. Levels for 3α and 5α were far lower than those at LRR ($p < 0.001$), while levels were not distinguishably different at the two endpoints for M_0, M_1, and M_2 ($0.1 < p < 0.9$).

Because the metabolites of 3α are present in substantial amounts, the possibility that they contribute to activity is of interest. With respect to M_0, M_1, and M_2, this does not seem to be the case because their levels were essentially the same at LRR, RRR_1, and RRR_2 (Figs. 1B and 2B). While 5α levels were markedly lower at RRR_2 than at LRR and therefore might have contributed to the behavioral changes, evidence from studies with mice (7) that 5α has lower potency than 3α makes this seem unlikely.

In Fig. 3A, it is illustrated that 3α levels at tLRR have a significant effect upon 3α levels at RRR_1. For the population (RRR_1) described in Fig. 2B, 3α levels at RRR_1 were subdivided into three groups on the basis of their tLRR values. Those that had the shortest tLRR (I) had 3α values (means ± SEM) significantly lower than those that had the longest tLRR (II, $p < 0.05$). The corresponding times (mean ± SEM) to reach RRR_1 for the two groups did not differ significantly. When 3α levels at RRR_2 (Fig. 2B) were similarly grouped, the same pattern was present although the difference in mean levels for the two groups was not significant. In Fig. 3B, 3α levels are shown for each of the three tLRR groups (highest, middle, and lowest) as functions of $tRRR_1$ and $tRRR_2$.

As shown in Fig. 3B, 3α levels at RRR_1 within each group remained approximately unchanged with increasing recovery time, with the exception of the two tadpoles with the longest $tRRR_1$ from the group with the highest levels (triangles). Total levels within each group were also approximately unchanged with increasing $tRRR_1$ (not shown).

From Fig. 4A, it is seen that total steroid levels were approximately constant for the first 6 min after transfer to water following tLRR. Total steroid levels at 3 min and 6 min after tLRR were assayed and then expressed as ratios to the levels at the corresponding tLRR. The latter were determined for each tadpole by interpolation from a standard curve of total steroid levels vs. tLRR.

Rates of Loss of Steroids from Brain

At RRR_2, 3α levels and total steroid levels (T) were approximately 24 and 63%, respectively, of levels at RRR_1 (Fig. 4B). The loss rate of total steroids from brain during the period from $tRRR_1$ (8 ± 2 min, mean ± SEM; $n = 13$) to $tRRR_2$ (45 ± 8 min, $n = 11$) was approximately 0.3 pmol mg^{-1} min^{-1}; correspondingly, the loss rate of 3α was about 0.15 pmol mg^{-1} min^{-1}. To the extent that steroid continues to enter the brain from the blood during this period, the actual rates of loss would be somewhat higher. The percentage decline of 3α levels was twice that of total steroid levels. The difference between the two is the result of the additional contribution to decline due to conversion of 3α to metabolites. The slow rates at which T and 3α change following the transfer to water (Fig. 4) are consistent with observation that steroid levels measured at LRR and RRR_1 were not significantly different (Figs. 1-3).

DISCUSSION

Critical Compartment

The evidence that the target sites in the tadpole can distinguish between closely related steroids such as 3α and its metabolites (Fig. 1) or 3α and PG (6) supports a receptor hypothesis and is also consistent with evidence obtained from studies of mice (6,7). Following Ariëns and Rossum (1), we suggest that LRR (an all-or-none behavioral response) occurs when the level of 3α in a compartment (C) that contains the target sites reaches a trigger level (L_T) that exceeds a fixed value ($3\alpha_0$). The trigger level is a function of receptor concentration, receptor occupancy, and $3\alpha_0$. This model is compatible with the one elaborated by Eyring et al. (3) or Franks and Lieb (4), but it is not compatible with models based upon nonspecific interactions at membrane target sites (12,13,16). The essential

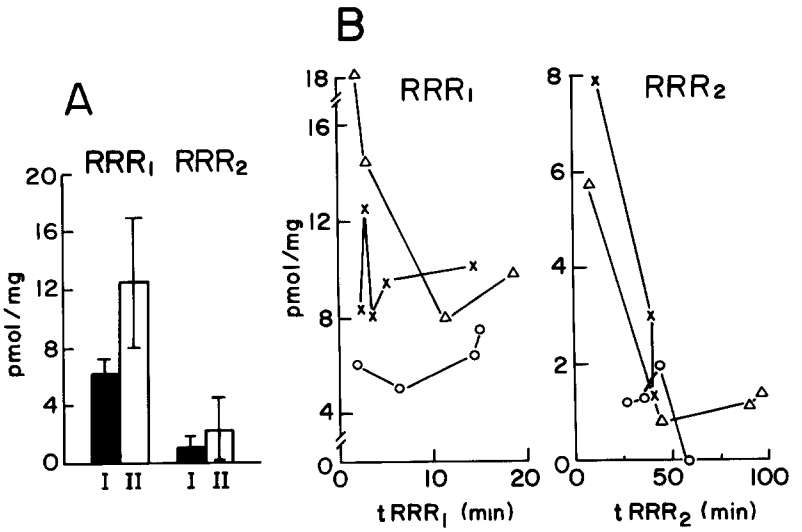

FIG. 3. Levels of 3α in tadpole brain at RRR_1 and RRR_2. (A). 3α levels (mean ± SEM) at RRR_1 were compared for groups that had reached LRR at (I) the shortest times (14–16 min; $n = 4$) and at (II) the longest times (30–48 min; $n = 4$). Similarly, at RRR_2 3α levels were compared for the groups that reached LRR at (I) the shortest times (9–18 min; $n = 4$) and at (II) the longest times (31–61.5 min; $n = 4$). (B). Levels of 3α (mean ± SEM) at $tRRR_1$ and $tRRR_2$. (○), tadpoles from group I; (△), tadpoles from group II; (×), the group with tLRR that are between I and II. 3α, 3α-hydroxy-5α-pregnan-20-one.

unsolved problem remains that of the identity of the compartment that contains the target sites and L_T.

The Whole Brain Is Not the Critical Compartment

Very different 3α levels were observed in whole brain depending upon whether PG or 3α was the administered agent. RRR_1 occurred without a decline in whole brain 3α levels (Figs. 1 and 2B) as would have been expected if the total 3α levels were critical to activity. Aqueous phases in brain contain far less 3α than membranous phases because partition behavior favors the membrane (~3,000:1) (10). Changes in 3α levels within an aqueous subcompartment are therefore unlikely to be detected by measurements of whole brain 3α levels. This highlights the possibility that the critical compartment is an aqueous phase. If this is the case, 3α levels in whole brain can be considered to provide only an index of 3α levels in the critical compartment.

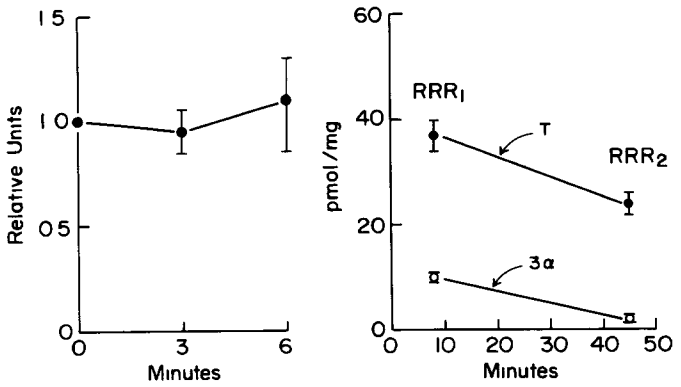

FIG. 4. Time course for steroid levels after LRR. (A). Tadpoles were incubated with 3α and killed at tLRR or after transfer to water at tLRR + 3 min and at tLRR + 6 min. Total steroid levels in brain were normalized relative to that at LRR ($n \geq 3$) and are plotted in relative units. (B). Decline in steroid levels. T, total steroid (●); 3α, 3α-hydroxy-5α-pregnan-20-one (○).

RRR_1 and RRR_2

The neuronal circuits that mediate RRR in response to 3α levels may be the same as those that mediate LRR. However, alternatively sensory or volitional contributions through other independent circuits may also override the activity of 3α at its receptors, or it may be that desensitization of occupied receptors leads to RRR_1 and/or RRR_2. In these alternative cases, the measured differences in 3α levels at RRR_1 and RRR_2 would not have functional significance.

During the period of 3α uptake, the conversion of 3α to metabolites slowed the onset of tLRR. In contrast, following the transfer to water the conversion of 3α to metabolites tended to reduce the 3α level and might encourage the regain of the righting response. Losses in 3α levels (and total levels) just following the transfer to water were almost nil and were probably balanced by gains from the blood. The decrease in 3α levels with time are predominantly due to metabolism. Even at longer times (8–45 min), the rate of loss of all steroids from the brain was slow (approximately 0.3 pmol mg^{-1} min^{-1}).

Although determinations related to time can be made for all three behavioral endpoints (LRR, RRR_1, and RRR_2) on a single tadpole, steroid levels can only be determined at a single endpoint because the tadpole must be killed to make the determinations. Because variations among individual tadpoles are large (Fig. 1A), comparisons between levels at different endpoints require comparisons between groups of tadpoles. It is

also the case that RRR_2 must be assessed to identify the complete population of RRR_1 because tadpoles do not always exhibit RRR_1. If the assay for RRR_2 were not carried out, tadpoles that failed to show a righting response corresponding to RRR_1 would be falsely scored as RRR_1 when they did finally exhibit a righting behavior corresponding to RRR_2. The results of Figs. 2–4 would be biased in that case.

Future experiments will explore the hypothesis that steroid receptors are contained in or accessed from an aqueous subcompartment of whole brain.

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